THE BIOCHEMICAL JOURNAL

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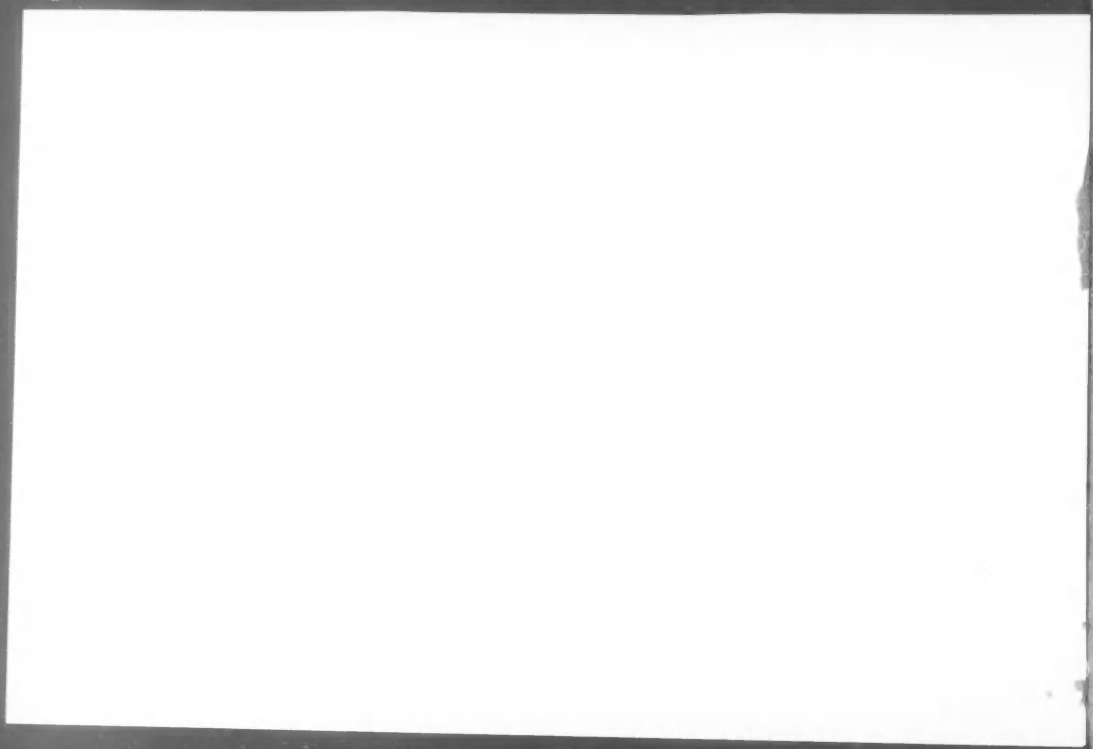

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Synthesis of L-Ascorbic Acid in Plants and Animals

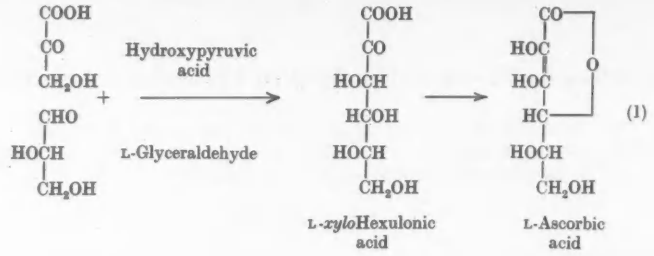
By F. A. ISHERWOOD, Y. T. CHEN and L. W. MAPSON
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research

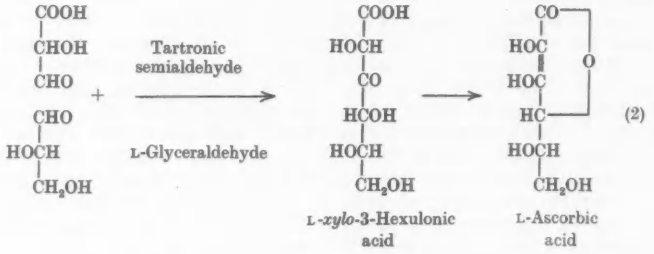
(*Received* 13 *May* 1953)

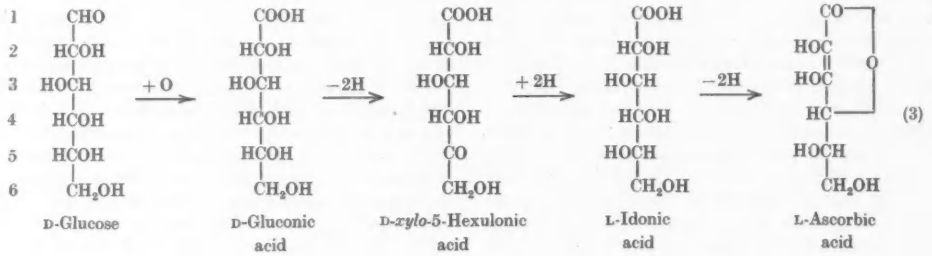
The chemical synthesis of L-ascorbic acid from D-galactose by Haworth & Hirst (1933) forms an outstanding achievement in the chemistry of the carbohydrates, and has frequently stimulated speculation as to whether the biological synthesis of L-ascorbic acid proceeds along similar lines. The very common occurrence of sugars in both plants and animals, and the fact that L-ascorbic acid is closely related to a sugar, L-gulose, has tended to give support to this idea. The idea has not hitherto been supported by any extensive body of facts, although there is a strong suggestion from earlier work that hexose sugars provide the carbon skeleton of the molecule. A number of experiments have been described in the literature which suggest that the carbon skeleton of L-ascorbic acid in both plants (Ray, 1934) and animals (Ruffo & Tartaglione, 1948; Jackel, Mosbach, Burns & King, 1950) is formed from a hexose sugar, though none of them definitely identified any one sugar as the precursor. In the case of plants, some workers (Ray, 1934; Tadokoro & Nisida, 1940; De & Barai, 1949) have claimed that D-mannose is the precursor, whilst others (Sugawara, 1941; Ahmad, Qureshi, Babbar & Sawhney, 1946; Itô & Mizuno, 1948) have suggested that a number of sugars can act as precursors. Such conflicting claims are not unexpected in view of the large variety of plant materials which have been examined. The hexose sugars and probably a number of other compounds such as sorbitol and inositol are interconvertible, but to different extents in different plants, so that the success obtained by feeding any of these compounds may well depend on whether the plant can convert them into the actual precursor of L-ascorbic acid. It is perhaps significant that with every kind of plant material examined D-glucose has caused the formation of L-ascorbic acid, whereas the other sugars, D-mannose, D-galactose and L-sorbose, have in a number of experiments given negative results (Itô & Mizuno, 1948; Devyatnin, 1950; Mapson, Cruickshank & Chen, 1949). In the case of animals the work of Jackel *et al.* (1950) has shown that a direct link exists between D-glucose and L-ascorbic acid though the evidence does not eliminate the possibility that D-glucose may be converted into D-mannose or D-fructose before being transformed into L-ascorbic acid; feeding D-[^{14}C]glucose labelled uniformly in all positions to chloretone-treated rats gave L-ascorbic acid which was also labelled uniformly in all positions. Mosbach and co-workers suggest that the carbon chain of the D-glucose is not broken before being converted to L-ascorbic acid or, if so, the fragments are recombined without any major differential dilution effect. No clue is given in any of the above work as to the series of changes by which the D-glucose is transformed into L-ascorbic acid.

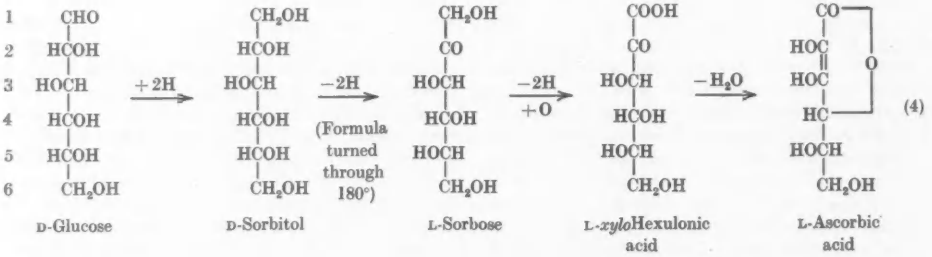
The observation that there is a close link between a hexose sugar, which may be D-glucose, and L-ascorbic acid has stimulated speculation as to the method by which the transformation from one to the other is effected, particularly the means by which a member of the D-series of sugars is changed to a member of the L-series. The investigations to be described in this and subsequent communications are directed towards establishing the nature of the hexose precursor and elucidating the mechanism by which the hexose is transformed into L-ascorbic acid; and the present paper describes evidence in support of a suggested mechanism for the transformation of two common hexoses, D-glucose and D-galactose, into L-ascorbic acid by a series of simple reactions which could reasonably be imagined as occurring in biological systems. Various possible sequences of reactions have been examined in which the main emphasis has been on changes in configuration necessary for the sugar to be transformed into L-ascorbic acid and in which no attempt has been made to portray the sugars or sugar acids in their cyclic forms, even though in practice the sugar acids were fed or injected as lactones in order to facilitate their entry into the cell. In every sequence of reactions the assumption has been made that the starting sugar is D-glucose though, in the brief discussion which follows, an indication has been given as to whether D-galactose or D-mannose could replace D-glucose.

The schemes described represent only a few of the many which on chemical grounds can be suggested for the transformation, but they serve to focus attention on the type of compounds which might be precursors of L-ascorbic acid. For convenience in presentation they have been grouped under two main headings.









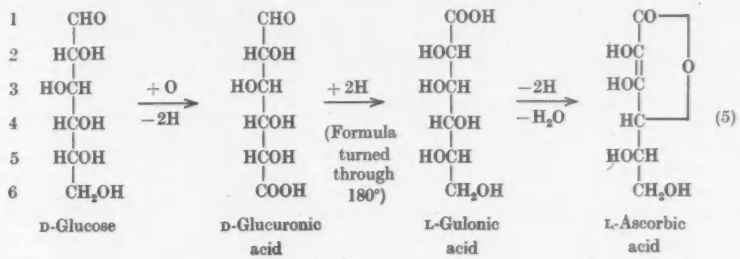
Indirect conversion. Indirect conversion, in which the carbon chain is broken and then reformed, involves the breakdown of the D-glucose via fructose diphosphate to the triose phosphates and the conversion of these into other three-carbon compounds, which then combine to give L-ascorbic acid. Assuming that this occurs, two types of condensation reaction can be suggested.

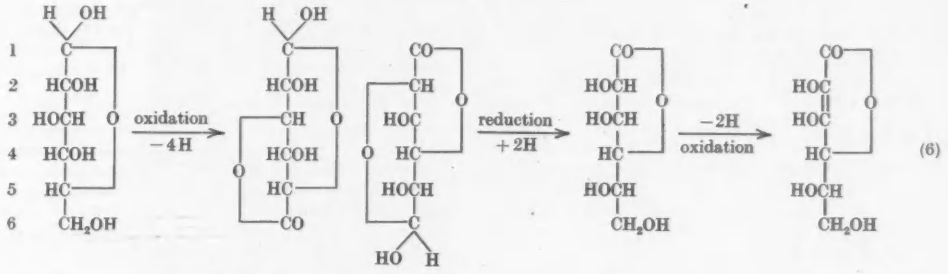
The first (1) is based on an aldol condensation between L-glyceraldehyde and hydroxypyruvic acid (Hough & Jones, 1951, 1952) catalysed by an enzyme similar to the aldolase of animal tissues (Meyerhof, Lohmann & Schuster, 1936) and plant tissues (Stumpf, 1948).

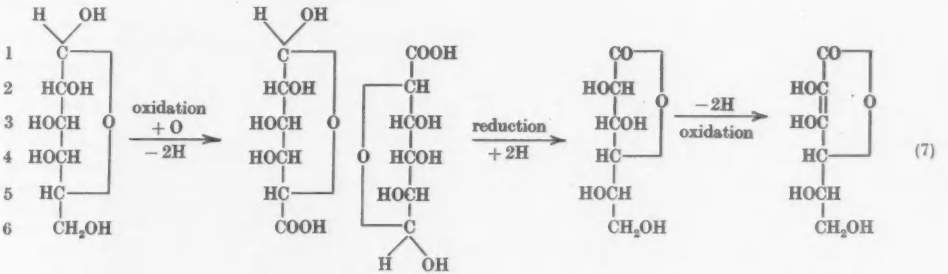
The second type of condensation (2) is an acyloin reaction between tartronic semialdehyde and L-

glyceraldehyde. L-*xylo*-3-Hexulonic acid (3-keto-L-gulonic acid) is known to be in tautomeric equilibrium with L-ascorbic acid, the equilibrium being very much in favour of the L-ascorbic acid (Cox, Hirst & Reynolds, 1932).

Some support for the general idea of indirect conversion came from the observation, which is described at length in another paper, that the formation of the three-carbon compound D-glyceric acid ran parallel with the formation of L-ascorbic acid in germinating cress seedlings and in rats. However, D-glyceric acid does not appear to be a precursor, for feeding the ethyl esters of D- or DL-glyceric acid to cress seedlings or injecting the compounds into rats did not produce any increase in the synthesis of L-ascorbic acid. It seems possible that the parallel formation of D-glyceric acid and L-ascorbic acid is due to a common cell environment rather than to a direct link between the two compounds and may therefore be misleading.







Direct conversion. The observation of Jackel *et al.* (1950) made it seem possible that the conversion of hexose into ascorbic acid takes place without rupture of the carbon chain. This must involve either the inversion of the configuration of the groups on carbon atom 5, or what amounts to an inversion of all the asymmetric carbons as written by transferring in effect the aldehyde or potential aldehyde group from carbon atom 1 to the terminal atom of the carbon chain. Thus the reduction of $C_{(1)}$ of an aldohexose to $—CH_2OH$ and the oxidation of $C_{(6)}$ to an aldehyde or potential aldehyde would mean that if the formula is now written according to the usual convention (being turned through 180° so as to bring the new aldehyde group at the top) the stereochemical designation of each hydroxyl group (D or L) is necessarily reversed. In the case of D-glucose the hydroxyl group on the new $C_{(5)}$ (old $C_{(2)}$) is now in the correct orientation for L-ascorbic acid.

The inversion of the groups attached to carbon atom 5 could occur through the intermediate formation of a 5-oxo compound, for example as in (3). In such a scheme the configuration of the groups on carbon atoms 2 and 3 is immaterial because in L-ascorbic acid they are not optically active. The configuration of the hydroxyl on carbon atom 4 must be D. This is true of D-mannose but not D-galactose.

The possibility of transferring the potential aldehyde group from carbon atom 1 to the terminal atom of the carbon chain has been thoroughly investigated in the present study. One scheme which illustrates the idea (4) is very similar in outline to the sequence of reactions used for the commercial manufacture of L-ascorbic acid. The configuration of the hydroxyls on carbon atoms 2 and 3 must be D and L, respectively. This is true of D-galactose but not D-mannose.

A further scheme of the same general type is (5). Support for this came from the observation of Smythe & King (1942) that treatment of rats with various drugs (e.g. chloretone) increased the excretion of both D-glucuronic acid (as glucuronide) and L-ascorbic acid. D-Galactose but not D-mannose could replace the D-glucose.

This scheme appears to be fundamentally the correct one. It can be written more conveniently in terms of the derivatives which were actually used to test its applicability to rats and cress seedlings. Scheme 5 for glucose and galactose can then be written as (6) and (7), respectively, which illuminates, in a striking manner, the crucial inversion from the D- to L-configuration by a series of plausible biochemical reactions.

The chief purpose of this paper is to present evidence that the reaction pathways of these last two schemes actually exist in plant and animal tissues. Briefly, the evidence consists of the demonstration that of a large number of closely related sugar derivatives, four sugar acid derivatives, L-gulono-γ-lactone, L-galactono-γ-lactone, D-glucurono-γ-lactone and D-galacturonic acid methyl ester, when fed to germinating cress seedlings or injected into rats, produced a very marked increase in the synthesis of L-ascorbic acid. These compounds are precisely those which should have an effect if the reactions in the above schemes (6) and (7) described the path by which L-ascorbic acid is synthesized; and moreover, no other closely related compound gives rise to L-ascorbic acid.

METHODS

The possibility that the above schemes outline the pathway by which L-ascorbic acid is synthesized from D-glucose has been examined by feeding various compounds postulated as intermediates in schemes 1–5 to cress seedlings, or injecting them into rats, and by comparing the formation of L-ascorbic acid with that in untreated controls. In the case of rats the tissues are normally saturated with L-ascorbic acid so that they excrete in their urine a small approximately constant amount which can be used as a measure of the synthesis of L-ascorbic acid in the intact animal. Any increase in the synthesis of L-ascorbic acid in the tissues is immediately reflected in the urinary excretion. The experiments on cress seedlings and on rats will be described separately.

Before discussing the significance of the results obtained, it is important to emphasize that certain of the compounds fed were simple derivatives of those originally postulated in schemes 1–5. These derivatives were chosen because they would penetrate into the cell, whereas those originally suggested would not do so, and yet once inside the cell would decompose to give the postulated intermediate. In the present study it was considered essential to make sure that the compound had at least penetrated into the seedling itself. Even then, the possibility remained that it might be unable to reach the active centres of synthesis, but for the present study the assumption was accepted that if the compound was present in the sap, it was available for conversion into L-ascorbic acid. Our experience was that organic compounds (Simon & Beevers, 1951) which were ionized in solution penetrated into the cress seedlings little if at all, whereas compounds which were un-ionized in solution, such as esters, lactones and sugars, penetrated readily (Beevers, Goldschmidt & Koffler, 1952). Acids such as galacturonic were not absorbed, but if they were fed as the methyl or ethyl esters, these penetrated into the seedling and then hydrolysed to give the free acid in the sap. α-Hydroxy acids such as galacturonic can be very conveniently handled in this way because their esters usually hydrolyse fairly rapidly in aqueous solution. Sugar acids such as D-gluconic, D-mannonic, L-gulonic and L-galactonic were fed as the γ-lactones. These were chosen because they were internal esters and were therefore free from the criticism that a 'foreign' alcohol was being introduced into the cell which might affect the synthesis of L-ascorbic acid; in aqueous solution they hydrolyse very slowly and might therefore be expected to penetrate into the cell in the same way as the esters.

Preparation of chemicals

Many of the chemicals used in this study were not obtainable commercially and had to be synthesized. Details are given in Table 1.

Each of the compounds described was examined on a paper chromatogram using as solvent either the water-poor phase from a two-phase mixture of *n*-butanol:glacial acetic acid:water (4:1:5 by vol., respectively) or a single phase solvent *n*-propanol plus concentrated ammonia (7:3 by vol. respectively). In the latter solvent the lactones were decomposed and moved as the ammonium salts of the free acids. In all cases except that of L-gluconic acid the compounds were free from other sugar derivatives. Some of the γ-lactones tended to decompose even with the acid solvent to give small amounts of the free acid and the δ-lactone, but these were readily identified.

Analytical technique

Many of the experiments which will be described later depended upon the detection, and in some cases the quantitative estimation, of minor constituents of the cell sap. For some, chemical methods were available, but for many the only method was the examination of the cell sap on a paper chromatogram. A full description of the apparatus and the general conditions of working in order to obtain the maximum resolution of the solutes on the paper chromatogram has been given in an earlier study on the separation of the sugars from plant extracts by Isherwood & Jermyn (1951). In the present paper only the modifications necessary for the separation of the particular compounds under investigation will be described. In many cases the quantitative chromatographic methods supplemented the results obtained by older chemical methods. Analysis of the urine

Table 1. *Preparation and properties of sugar acid derivatives*

(All but one are γ-lactones. Rotations were in water unless specified otherwise and were at room temperature.)

Substance (γ-lactone)	Method of preparation	References	M.p. (°) (uncorr.)	$[\alpha]_D$ (°) (*c* in parentheses)	Notes
D-Allono	From D-ribose (cyanohydrin)	(*a*)	130	−6 (3·5)	—
D-Altrono	From D-ribose (cyanohydrin)	(*a*)	Syrup	+35 (2·7)	Free acid prepared from Ca salt by addition of HCl; $[\alpha]_D$ +8 (4)
D-Gulono	From D-xylose (cyanohydrin)	(*b*)	180	−56 (1)	—
D-Idono	From D-xylose (cyanohydrin)	(*b*) (*c*) (*d*)	Syrup	−53 (10)	Brucine salt of acid, m.p. 182°. Dibenzal derivative, m.p. 204°
D-Mannono	From D-mannose by oxidation	(*e*)	150–152	+51·5 (1)	—
D-Glucono	From D-glucose by oxidation	(*e*) (*f*)	133–136	+68 (5)	—
D-Galactono	From D-galactose by oxidation	(*e*)	132–133	−73 (5)	—
D-Talono	From D-galactonic acid by epimerization	(*g*)	130	−33 (2)	Free acid, m.p. 125°; $[\alpha]_D$ +17 (5)
L-Gulono	From D-glucuronic acid by reduction with Na/Hg	(*h*)	180	+55 (1)	—
L-Idono	From L-gulonic acid by epimerization	(*c*)	Syrup	+50 (5)	Brucine salt of acid, m.p. 190–192; $[\alpha]_D$ −17 (5)
L-Galactono	From D-galacturonic acid by reduction with Na/Hg	(*h*)	132	+75 (5)	—
L-Talono	From L-galactonic acid by epimerization	(*g*)	130	+33 (2)	Free acid, m.p. 125°; $[\alpha]_D$ −17 (5)
L-Mannono	From L-arabinose (cyanohydrin)	(*b*)	150	−51 (5)	—
L-Glucono	From L-arabinose (cyanohydrin)	(*b*)	—	—	Crude syrup after removal of bulk of L-mannono-γ-lactone
D-Mannurono	From alginic acid	(*i*)	138	+92 (3)	—
D-Galacturonic acid methyl ester	From D-galacturonic acid	(*j*)	145	+94 (2, MeOH)	—

References: (*a*) Phelps & Bates (1934); (*b*) Hudson, Hartley & Purves (1934); (*c*) Fischer & Fay (1895); (*d*) Micheel & Kraft (1933); (*e*) Hudson & Isbell (1929); (*f*) Isbell & Frush (1933); (*g*) Glatthaar & Reichstein (1938); (*h*) Thierfelder (1891); (*i*) Frush & Isbell (1946); (*j*) Jansen & Jang (1946).

of the rat was effected by essentially the same methods as those used for the cress seedling.

The analysis consisted mainly in determinations of L-ascorbic acid, sugars and organic acids such as D-glyceric acid.

L-Ascorbic acid was estimated chemically by oxidation with 2:6-dichlorophenol indophenol, the procedure adopted being essentially that described by Harris & Olliver (1942). The quantitative chromatographic method described by Chen, Isherwood & Mapson (1953) will distinguish between D-*arabo*ascorbic acid and L-ascorbic acid and was used particularly in connexion with experiments in which various sugar acid derivatives were fed to cress seedlings or injected into rats.

Fermentable and non-fermentable sugars were estimated by the method of Mapson, Cruickshank & Chen (1949) or by the chromatographic method of Jermyn & Isherwood (1949). The accuracy of the latter method has been much improved by a modification to the copper reagent used for estimating the separated sugar. Wager (private communication) has found that by carrying out the oxidation of the sugar in the absence of oxygen, the precision of the results with small amounts of sugar is very much increased.

In the qualitative examination of the cell sap of the cress seedling, the amount of acid present was judged visually from the size of the spot on the paper chromatogram using solvents recommended by Isherwood & Hanes (1953). This was particularly useful in the examination of cress seedlings which had been fed with various sugar-acid derivatives, when it was essential to know whether the acid had penetrated into the seedling and whether it had noticeably affected any of the other constituents. Further details of the qualitative examination of the cress seedlings will be given later in the description of the separate experiments.

Culture of cress seedlings

The procedure used varied slightly as the work progressed but the preferred method was as follows.

About 300 seeds were placed on a piece of fine Nylon fabric (area about 60 cm.^{2}) which covered the bottom of a white porcelain jar. Underneath the Nylon fabric was a piece of filter paper of about the same size. Sufficient of the appropriate solution was then added so that the seeds were just wetted. Nylon fabric was chosen because it is not absorptive, and if necessary it can be lifted out of the jar with the seedlings still in position, fresh solution and filter paper introduced, and the Nylon replaced in its original position. This is particularly useful when the aqueous solution slowly decomposes as with the sugar acid lactones and esters and it is essential to feed the seedlings with fresh solution each day. The seeds were germinated in the dark at a temperature of 20° and the jars were covered to prevent evaporation.

For analysis the seedlings were usually washed to remove any adherent solution and then the testas removed. The subsequent treatment depended upon whether they were to be analysed for L-ascorbic acid or whether the constituents of the cell sap were to be examined on a paper chromatogram. For the latter they were frozen at −20° and held at this temperature until required.

Treatment of rats with various compounds

Piebald male rats of varying ages but usually about 140 g. in weight were given a basal diet containing casein 20, sucrose 60, arachis oil 15, and salt mixture 5%, with an additional 10% of dried yeast. Each animal received weekly supplements of vitamins A and D by the administration of two drops of halibut-liver oil (=1500 i.u. of A and D/rat/week, respectively). Vitamin E was given as 1 mg. of (±)-α-tocopherol acetate. The rats were housed in metabolism cages in which all metal parts coming into contact with urine were waxed. The urine was collected in beakers containing 10 ml. of 10% (w/v) oxalic acid to avoid any decomposition of the L-ascorbic acid. The rats were rested until their urinary excretion of L-ascorbic acid was steady and then they were injected subcutaneously with 0·5 ml. of an aqueous solution of the compound to be tested. Commonly 100 mg. of the organic compound was injected.

RESULTS

Experiments with cress seedlings. The effect on the synthesis of L-ascorbic acid of feeding various sugar derivatives

In the experiments described in Table 2, compounds which were postulated as intermediates in various possible sequences of reactions linking glucose with L-ascorbic acid were fed to cress seedlings and the formation of L-ascorbic acid in these seedlings compared with that in seedlings grown in water under identical conditions of temperature and humidity; a fresh control experiment on seedlings grown in water was made for each compound.

The results described in Table 2 show that feeding the γ-lactone of D-gluconic acid (scheme 3), L-sorbose and D-sorbitol (scheme 4) and DL-glyceraldehyde (schemes 1 and 2) did not cause any marked increase in the synthesis of L-ascorbic acid as compared with that in seedlings grown in water. Experiments with D-xylose and L-arabinose were included to show the results obtained when compounds were fed which were neither metabolized by the seedlings to any extent, nor likely to be precursors of L-ascorbic acid. These experiments formed a very useful standard by which to judge the other experiments. Thus it would appear that the synthesis of L-ascorbic acid does not proceed through any of the compounds mentioned above, though it must be emphasized that if phosphorylated derivatives of the compounds were the actual intermediates, then the compounds fed to the seedlings would have to be phosphorylated inside the cell before becoming available for conversion into L-ascorbic acid. This possibility would tend to make any conclusions which might be derived from these feeding experiments rather tentative. However, the striking results obtained by feeding under analogous conditions the γ-lactones of D-glucuronic and L-gulonic acids (scheme 5) diminish the importance of this criticism. These compounds definitely increased the synthesis of L-ascorbic acid even though the cell sap became more acid. The growth and appearance of the seedlings were practically indistinguishable from those grown in water.

Table 2. *Synthesis of* L-*ascorbic acid in cress seedlings grown in solutions of various sugar derivatives*

Sugar derivative	Duration of experiment (hr.)	Weight of 100 seedlings (g.)	L-Ascorbic acid as % of control grown in water	Examination* of cell sap
D-Glucono-γ-lactone (0·5%)	72	1·10	61	pH 4·56 C
	96	1·20	43	4·44
	120	1·60	60	
DL-Glyceraldehyde (0·1%)	48	1·0	82	
	72	1·73	87	
	96	2·51	89	C
	120	2·96	95	
	144	3·18	83	
D-Glyceraldehyde (0·1%)	72	1·0	93	
	96	1·4	108	C
	120	1·8	103	
D-Sorbitol (1%)	51	1·03	96	
	72	1·51	98	
	96	1·88	108	C
	120	2·08	102	
	144	2·44	113	
L-Sorbose (1%)	51	1·0	75	
	72	1·38	88	
	96	1·89	103	U
	120	2·14	100	
	144	2·21	103	
D-Glucurono-γ-lactone (1%)	72	1·00	97	
	92	1·30	125	
	118	1·80	127	C
	142	1·90	131	
	163	1·90	149	
L-Gulono-γ-lactone (1%)	72	1·30	152	pH 4·6 C
	96	1·60	139	4·59
	120	1·80	144	4·41
	142	2·10	137	
D-Xylose (1%)	72	1·0	74	
	96	—	100	U
	140	—	114	
	170	1·90	112	
L-Arabinose (1%)	72	0·96	82	
	96	—	100	U
	140	—	108	
	170	2·35	106	

* U, the cell sap contained an appreciable amount of the sugar derivatives as measured by the rise in the unfermentable sugars. C, examination on a paper chromatogram showed that the cell sap contained an appreciable quantity of the sugar derivative.

Feeding L-*xylo*hexulonic ester also appeared to increase the L-ascorbic acid content but this was later found to be due to the spontaneous conversion of the ester into L-ascorbic acid in aqueous solution, especially in the presence of alkaline buffers (pH 6–8). The L-ascorbic acid was readily absorbed by the seedlings and it thus appears likely that the observed increase was a result of a non-enzymic conversion of the ester into L-ascorbic acid. This experiment, which is not described in Table 2, therefore does not provide unequivocal evidence that the ester is a precursor of L-ascorbic acid in the seedling. In connexion with this discovery, it is interesting to note that it has been claimed that the ester has anti-scorbutic properties (Reichstein & Demole, 1936).

The experiment in which DL-glyceraldehyde was fed deserves closer study. Large concentrations of DL-glyceraldehyde inhibit growth but at the concentration employed in the experiment (0·1%, w/v) described in Table 2, growth was practically unaffected (weight 3·2 g./100 seedlings at 144 days as compared with 2·7–3·4 for seedlings grown in water). The L-ascorbic acid content, however, was definitely lower. This appears to be due to the presence of L-glyceraldehyde, for feeding D-glyceraldehyde in the same concentration did not affect the synthesis of L-ascorbic acid.

The interpretation of feeding experiments with the potentially acid compounds was sometimes complicated by the effect of the compounds on the pH of the cell sap apart from the actual synthesis of

the L-ascorbic acid. An example of this can be quoted from the results in Table 2. D-Glucono-γ-lactone (0·5 %, w/v, in water) fed to cress seedlings clearly depressed the formation of L-ascorbic acid (50 %) because the amount in the sap was sufficient (estimated from paper chromatograms to be about 0·5 % at the sixth day) to make it definitely more acid (pH 4·4) than that of seedlings grown in water (pH 4·8). A rough calculation of the amount by which the synthesis of L-ascorbic is depressed by a change in the pH of the sap can be made from the experiments described by Mapson *et al.* (1949). They showed that the synthesis of L-ascorbic acid in cress seedlings was definitely related to the pH of the sap and was depressed when the sap became more acid. From the figures given in their paper, the change in the pH of the cell sap (4·8–4·4) in the experiment with D-glucono-γ-lactone would be expected to depress the synthesis by about 50 %. The observed depression would therefore appear to be almost entirely related to the change in pH of the cell sap, and it seems unlikely that the compound has any specific inhibitory effect on the synthesis.

The results described in Table 2 suggest strongly that the synthesis of L-ascorbic acid from D-glucose proceeds by a series of reactions similar to those outlined in scheme 5. Later experiments have been concerned with confirming the special position of D-glucurono-γ-lactone and L-gulono-γ-lactone in the synthesis and in establishing the specificity of the various reactions involved.

Examination of the specificity of the reactions involved in the conversion of hexoses to L-*ascorbic acid*

Cress seedlings. Two reactions outlined in scheme 5 have been examined in detail. Various compounds closely related to D-glucuronic and L-gulonic acids have been fed to cress seedlings and the L-ascorbic acid content compared with that of seedlings grown in water. The results of these experiments have indicated that two hexoses may act as precursors of L-ascorbic acid, namely D-glucose and D-galactose, the sequence of reactions connecting them with L-ascorbic acid being very similar. This was to be expected on theoretical grounds since the configuration of these sugars is such that they can be transferred from the D- to the L-series of sugars by transposing the terminal groups of the molecule as outlined in scheme 5, and the L sugar derivatives produced have the right configuration for a precursor of L-ascorbic acid. This fact was briefly mentioned when scheme 5 was discussed earlier in this paper.

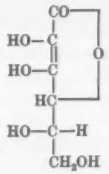
The group of compounds closely related to L-gulonic acid comprised all the known aldohexonic acids. In the feeding experiments, however, the γ-lactones of these were used and the configurations of these, written according to the usual cyclic modification of the Fischer convention, are shown in Fig. 1.

Only two compounds related to D-glucuronic acid (D-glucurono-γ-lactone was actually used) were fed to cress seedlings, namely D-mannurono-γ-lactone and D-galacturonic acid methyl ester. In Fig. 1 these have been drawn in such a way as to emphasize that they have a similar structural relationship to D-mannono- and L-galactono-γ-lactones, respectively, as D-glucurono-γ-lactone has to L-gulono-γ-lactone in scheme 5.

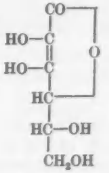
The formulae in Fig. 1 have been grouped so as to bring out as clearly as possible the relationship of the various lactones to L-ascorbic acid. Each horizontal line contains four lactones which have the same configuration as far as the carbon atoms 4 and 5 are concerned but differ between themselves as to the disposition of the groups on carbon atoms 2 and 3. No information can be obtained from the configuration of L-ascorbic acid as to the disposition of the groups on carbon atoms 2 and 3 of its precursor. In Fig. 1 the first line contains lactones which have the same configuration as L-ascorbic acid, the second line lactones in which the configuration of carbon atom 5 is opposite to that of L-ascorbic acid, and the third and fourth lines lactones which are enantiomorphs (mirror images) of the corresponding lactones in the first and second lines respectively.

The results of feeding the various sugar derivatives to cress seedlings are shown in Table 3. It is clear that L-gulono-, L-galactono-, D-altrono-, D-glucurono-γ-lactones and D-galacturonic acid methyl ester markedly increase the formation of an ascorbic-acid-like substance. This is particularly obvious if the results with these sugar derivatives are compared with those obtained with inactive lactones of very similar structure, e.g. D-gulono-, D-galactono- and D-mannurono-γ-lactones. There seems no doubt that all these lactones penetrated into the seedlings (the cell sap was examined on a paper chromatogram) and the difference in behaviour appears to be due to the specificity of the enzymes involved in the conversion to L-ascorbic acid.

The ascorbic-acid-like compound produced by feeding the cress seedlings with L-gulono-, L-galactono- and D-altrono-γ-lactones was separated from the other constituents of the cell sap and examined on a paper chromatogram by the method of Chen *et al.* (1953). Tracings of chromatograms from seedlings which had been fed with L-galactono- and D-altrono-γ-lactones are shown in Fig. 2. (The chromatogram from seedlings fed with L-gulono-γ-lactone has not been included because apart from the very much weaker concentration of the ascorbic-acid-like compound in the sap, the picture was essentially the same as for the L-galactono-γ-



L-Ascorbic acid



D-*arabo*Ascorbic acid

Types I and IV were γ-lactones of aldonic acids, Types V and VI were uronic acid derivatives with the same basic configuration as Types I and II, respectively.

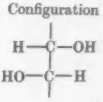
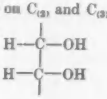
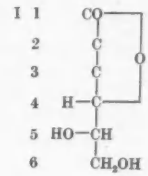
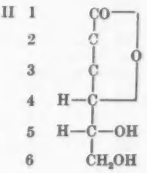
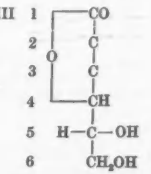
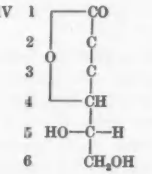
Type (same configuration of groups on $C_{(4)}$ and $C_{(5)}$)				
I 	L-GULONO*	L-Idono	L-Talono	L-GALACTONO*
II 	D-*Mannono** (rat)	D-Glucono	D-Allono	D-*Altrono** (cress)
III 	D-Gulono	D-Idono	D-Talono	D-Galactono
IV 	L-Mannono	L-Glucono	L-Allono (not examined)	L-Altrono
V As I but with —CHO at $C_{(6)}$ instead of $-CH_2OH$	D-GLUCURONO-* γ-LACTONE	—	—	D-GALACTURONIC ACID METHYL ESTER
VI As II but with —CHO at $C_{(6)}$ instead of $-CH_2OH$	D-Mannurono-γ-lactone	—	—	—

Fig. 1. Sugar acid derivatives fed to cress seedlings.

* Compounds in capital letters give L-ascorbic acid, whereas compounds in *italics* give D-*arabo*ascorbic acid, in the cress seedling and in the rat as shown.

Table 3. *Effect of feeding various sugar acid derivatives to cress seedlings on the formation of* L-*ascorbic acid*

(All solutions were put in after 48 hr. germination in water and changed every day. At the time of the first ascorbic acid determination the cell sap was, in every case, examined for the substance fed.)

Sugar derivative fed to seedlings*	Duration of experiment (hr.)	Apparent ascorbic acid as % of that in seedlings grown in water
D-Glucuronolactone	72	97
	96	105
	120	117
	144	123
D-Galacturonic acid methyl ester	72	144
	96	140
	140	172
	164	171
D-Mannuronolactone	96	72
	120	58
	148	57
L-Gulonolactone	72	95
	96	137
	120	146
	144	148
L-Idonolactone	72	103
	96	62
	120	46
L-Talonolactone	72	83
	96	84
	120	69
	144	75
L-Galactonolactone	72	320
	96	256
	120	357
	144	482
D-Mannonolactone	72	102
	96	85
	120	77
	144	79
D-Gluconolactone	72	59
	96	47
	120	55
D-Allonolactone	72	97
	96	77
	120	78
	144	61
D-Altronolactone	72	387 †
	96	552 †
	120	580 †
D-Gulonolactone	72	70
	96	34
	120	27
D-Idonolactone	72	97
	96	56
	120	57
D-Talonolactone	72	127
	96	92
	120	78
	144	77
D-Galactonolactone	72	55
	96	60
L-Mannonolactone	72	76
	96	41
	120	43
L-Gluconolactone	96	64

* All 0·5% (w/v) except D-glucurono- and L-gulono-lactones (1%). † D-*arabo*Ascorbic acid produced.

lactone). Comparison of these chromatograms indicated that feeding L-galactono-γ-lactone increased the L-ascorbic acid content of the seedlings, and feeding D-altrono-γ-lactone increased the D-*arabo*ascorbic acid content.

The identification of these compounds as L-ascorbic acid and D-*arabo*ascorbic acid is admittedly not conclusive because the enantiomorphic forms would behave similarly on the chromatogram. However, if the configurations of the lactones which act as precursors in the cress seedling are taken into consideration, this uncertainty is removed; L-gulono- and L-galactono-γ-lactones can produce only L-ascorbic acid, and D-altrono-γ-lactone, D-*arabo*ascorbic acid.

Before discussing the results with cress seedlings, the comparable experiments with rats will be described.

Rats. The results of a number of the experiments on rats are shown in Fig. 3. Most of the experiments in which the titration figure for urine remained unchanged have not been described. The same series of sugar-acid γ-lactones were examined as in the case of cress seedlings.

From Fig. 3 it is clear that L-gulono-, L-galactono, D-mannono and D-glucurono-γ-lactones and D-galacturonic acid methyl ester increase the titration figure. Examination of the urine showed that D-mannono-γ-lactone gave rise to D-*arabo*ascorbic acid, whereas the others all gave L-ascorbic acid. The chromatograms were very similar to those shown previously in Fig. 2 for cress seedlings. It was interesting to note that neither D-altrono-γ-lactone, which might give D-*arabo*ascorbic acid, nor L-idono- and L-talono-γ-lactones, which are very similar in structure to L-gulono- and L-galactono-γ-lactones, respectively, produced any increase in the titration figure. D-Mannurono-γ-lactone which by analogy with D-glucurono-γ-lactone in scheme 5 should give D-mannono-γ-lactone on reduction

does not give D-*arabo*ascorbic acid. D-Mannono-γ-lactone has been shown previously to give D-*arabo*-ascorbic acid. It seems that the enzyme system which converts the uronic acid to the corresponding aldonic acid cannot convert D-mannurono-γ-lactone into D-mannono-γ-lactone.

The four sugar-acid derivatives which have been shown to act as precursors of L-ascorbic acid in cress seedlings and rats can be regarded as intermediates in two hypothetical series of reactions linking the D-hexoses with L-ascorbic acid. The first of these links D-glucose, D-glucuronic acid and L-gulonic acid to L-ascorbic acid and the second, which is very similar to the first, links D-galactose, D-galacturonic acid and L-galactonic acid to L-ascorbic acid. The only difference between the intermediates in the two series of reactions is the disposition of the groups on carbon atom 4 (carbon atom 3 if based on L-ascorbic acid), those in the D-glucose series having the hydroxyl on the right-hand side (formula written with the aldehyde group at the top) and those in the D-galactose series having it on the left. Although the experimental evidence did not allow a decision to be made as to whether D-glucose or D-galactose was the precursor, it was clear that the enzymes catalysing the series of reactions in the animal were not exactly the same in the rat as those in the plant, because their behaviour towards lactones which might form D-*arabo*ascorbic acid was markedly different. Feeding D-altrono-γ-lactone to cress seedlings gave D-*arabo*ascorbic acid but injected into rats had no effect, whereas feeding D-mannono-γ-lactone to cress seedlings had no effect but injected into rats gave D-*arabo*ascorbic acid. A possible explanation for these observations is based on the similarity in structure of L-galactono- and D-altrono-γ-lactones, and L-gulono- and D-mannono-γ-lactones, respectively (see Fig. 1). Each pair of lactones has the same ring structure and differs only in the disposition of the groups in carbon atom 5. It would appear that the enzyme in cress seedlings which converts either L-gulono- or L-galactono-γ-lactones into L-ascorbic acid, reacts more readily with the L-galactono-γ-lactone ring structure, whereas the corresponding enzyme in the rat reacts more readily with the L-gulono-γ-lactone ring structure. This specificity is only shown when an 'unnatural' lactone is present as substrate.

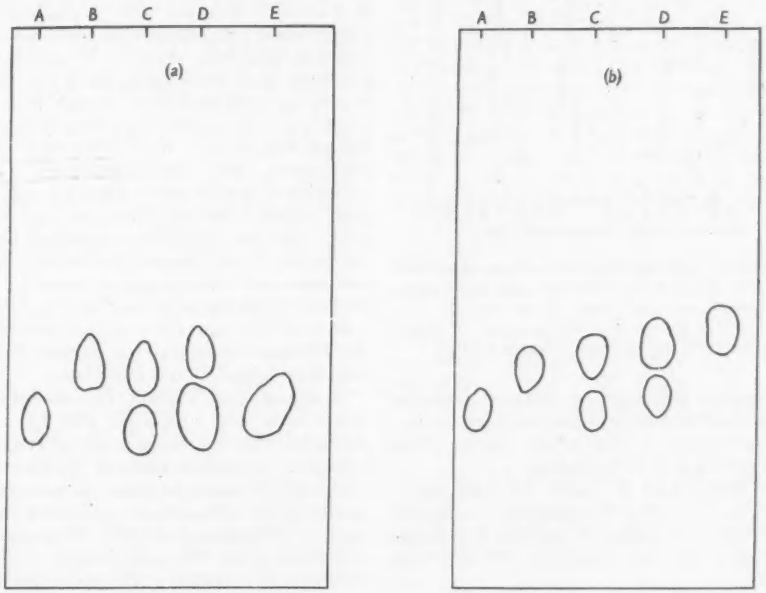


Fig. 2. Tracings of chromatograms showing the compounds present in the sap of cress seedlings which reduce 2:6-dichlorophenol indophenol. The cress seedlings were cultured either in a 0·5% (w/v) aqueous solution of D-altrono-γ-lactone (*a*), or in a 0·5% (w/v) solution of L-galactono-γ-lactone (*b*), for 5 days. Paper, Whatman no. 1. Solvent, phenol:water:oxalic acid. For comparison solutions (0·2%, w/v) of D-*arabo*ascorbic and L-ascorbic acids were spotted on the 'starting line' as shown. *A*, D-*arabo*ascorbic acid; *B*, L-ascorbic acid; *C*, L-ascorbic and D-*arabo*ascorbic acids; *D*, extract of seedlings, L-ascorbic and D-*arabo*ascorbic acids; *E*, extract of seedlings.

The substrate specificity of the enzyme catalysing the conversion of the aldonic acid γ-lactones to ascorbic-acid-like compounds deserves mention, because of sixteen possible precursors only four were active as substrates, two giving L-ascorbic acid. From the results described in Table 3 it is clear that the enzyme requires the disposition of groups on carbon atom 2 to be such that the hydroxyl is on the left-hand side of the molecule. L-Idono- and L-talono-γ-lactones, which are very similar to L-gulono- and L-galactono-γ-lactones, respectively, were without activity. These first two lactones have the hydroxyl on carbon atom 2 on the right-hand side.

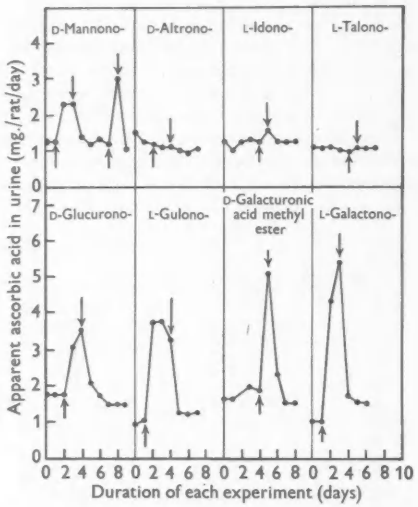


Fig. 3. Effect of injecting rats with various sugar acid derivatives on the excretion of ascorbic acid in the urine. The compounds were γ-lactones in all but one case. ↑ Marks the day the rats were first injected. ↓ Marks the day after which the rats were no longer injected.

The disposition of the groups on carbon atom 3 is not so important for both L-gulono and L-galactono-γ-lactones are active. In these the disposition of the groups on carbon atom 3 is opposite.

It seems essential for the ring to be on the right-hand side of the molecule (hydroxyl on carbon atom 4 on right-hand side), for only lactones which had the ring on the right-hand side were active as substrates.

The efficiency of the conversion of the aldonic acid lactone to L-ascorbic acid is difficult to estimate because it is probable that in the rat a high proportion of the lactone is excreted unchanged and in the cress seedling is converted to the free acid. L-Ascorbic acid (100 mg.) injected into the rat was excreted to the extent of about 60% and by analogy it is possible that a similar proportion of the sugar-acid lactones may be excreted unchanged. The observed ratio between the L-ascorbic acid in the urine and the lactone injected was approximately 3:100 in most of the experiments, though in one experiment when L-galactono-γ-lactone was injected as an aqueous suspension, the ratio was higher, 8:100. This suggests that the efficiency of the conversion might be higher if the lactone was fed continuously so as to maintain a low concentration in the blood, rather than injected all at once. In the case of cress seedlings the efficiency of the conversion has been estimated very roughly to be of the same order of magnitude. The main source of error lies in the fact that the amount of the lactone in the cell sap has been estimated visually from the size of the spot on a paper chromatogram.

CONCLUSIONS

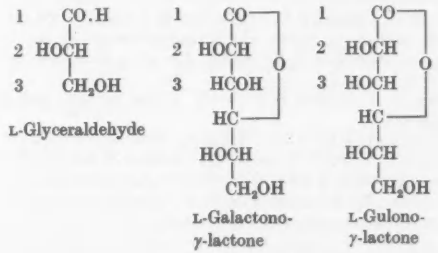
The foregoing experiments constitute a preliminary survey of the series of reactions which convert a hexose, which may be D-glucose or D-galactose, into L-ascorbic acid in both cress seedlings and the rat. Four compounds, L-gulono-, L-galactono- and D-glucurono-γ-lactones and D-galacturonic acid methyl ester, when fed to cress seedlings or injected into rats, have been shown to be transformed into L-ascorbic acid. The significance of this observation is very much increased by the fact that the other sugar-acid derivatives with closely related configurations such as L-idono- and L-talono-γ-lactones are inactive, except that two lactones, D-mannono-γ-lactone in the rat and D-altrono-γ-lactone in the cress seedling, are converted into D-*arabo*ascorbic acid. The four compounds described above have previously in this paper been postulated as intermediates in a hypothetical series of reactions (scheme 5) linking D-glucose (and D-galactose) with L-ascorbic acid, and the fact that these compounds can be transformed into L-ascorbic acid lends considerable support to this hypothesis.

It is possible to explain the conversion of these compounds into L-ascorbic acid by a series of reactions other than those described in scheme 5. In scheme 5 the carbon chain of the hexose was not broken but it is easy to imagine a series of reactions in which the carbon chain was broken down to C_2, C_3 or C_4 fragments and then resynthesized to give L-ascorbic acid. The experiments in the present study do not provide a rigid proof that the carbon chain of the hexose molecule remains unbroken but the earlier observations of Jackel *et al.* (1950) that feeding D-glucose uniformly labelled with ^{14}C to chloretone-treated rats gave L-ascorbic acid which was also uniformly labelled, also suggested that the carbon chain was not broken.

A critical test would be to feed D-glucose which was labelled only in one position along the carbon chain (carbon atom 1 or 6 would be convenient) to rats and then examine the distribution of the labelled carbon along the carbon chain of the resultant L-ascorbic acid. If the chain remained unbroken, the labelled carbon would only occur in the corresponding position along the carbon chain of the L-ascorbic acid. Recently two experiments of this type have been described, the results of which strongly support the reaction chain described in scheme 5. Administration of D-[1-^{14}C]glucose to chloretone-stimulated rats resulted in the urinary excretion of ascorbic acid containing ^{14}C chiefly in position 6 (Horowitz, Doerschuk & King, 1952). Similarly, administration of D-[6-^{14}C]glucose gave ascorbic acid containing ^{14}C in position 1 (Horowitz & King, 1953). The D-glucose must have been converted to ascorbic acid without breaking the carbon chain and a complete inversion of the configuration must have occurred.

In the present study two series of reactions (schemes 1 and 2) based on the condensation of L-glyceraldehyde with appropriate three-carbon compounds to give L-ascorbic acid were tested by feeding DL- and D-glyceraldehyde to cress seedlings. It was found that the formation of L-ascorbic acid was unaffected by feeding D-glyceraldehyde but was definitely depressed by the DL-glyceraldehyde. From these results it seemed unlikely that L-glyceraldehyde was concerned in the synthesis of L-ascorbic acid. The reduced formation of L-ascorbic acid by feeding DL-glyceraldehyde was interesting because L-glyceraldehyde (L-sorbose 1-phosphate is the active inhibitor, Lardy, Wiebelhaus & Mann, 1950) is known to inhibit the hexokinase enzyme present in animals and plants which catalyses the formation of D-glucose 6-phosphate from D-glucose.

If this were true of the hexokinase present in cress it would suggest that glucose 6-phosphate is concerned in the chain of reactions from D-glucose to L-ascorbic acid. However, another explanation of the inhibition can be suggested based on the similarity of the configuration of L-glyceraldehyde and the first three carbon atoms of L-gulono- and L-galactono-γ-lactones.



The resemblance is even closer than the formulae suggest because the carbonyl group at C-1 of the lactones has characteristic carbonyl properties which are absent when it exists as part of a carboxyl ion; presumably resonance in the group $C\begin{cases}O\\O\end{cases}H$ masks the true carbonyl properties in this case. The supposition is that the L-glyceraldehyde having a similar structure competes with the γ-lactones for the enzyme system which catalyses their oxidation to L-ascorbic acid.

The specificity of the enzymes catalysing the transformation of an aldonic acid to L-ascorbic acid requires that the hydroxyls on carbon atoms 2 and 5 (compared to L-ascorbic acid) shall have the L configuration, and that on carbon atom 4 the D configuration. An additional degree of specificity is implied by the fact that D-altrono-γ-lactone only in cress seedlings and D-mannono-γ-lactone only in the rat are converted into D-*arabo*ascorbic acid. This would suggest that the enzyme in the cress seedling reacts only with compounds which have a configuration for the first four carbon atoms similar to L-galactonic acid (D-altronic acid), whereas the enzyme in the rat reacts only with compounds similar to L-gulonic acid (D-mannonic acid). In the case of L-gulonic and L-galactonic acids, which as lactones give rise to L-ascorbic acid in both the rat and cress seedlings, it is conceivable that the two compounds can be converted into each other as in the enzymic conversion of D-galactose 1-phosphate to D-glucose 1-phosphate by a direct inversion of the groups at carbon atom 4 (Leloir, 1951; Topper & Stetten, 1951). The configuration of L-gulose and L-galactose closely corresponds to that of D-glucose and D-galactose, respectively (cf. scheme 5).

The specificity of the enzymes which catalyse the conversion of a uronic acid presumably via the related aldonic acid (scheme 5) to an ascorbic acid-like compound, has been tested in one case only. D-Mannuronic acid-γ-lactone on reduction gives D-mannonic acid, which (as the lactone) had earlier been shown to give D-*arabo*ascorbic acid in the rat. Injection of D-mannurono-γ-lactone into the rat did not affect the 2:6-dichlorophenol indophenol titration figure, so that it appeared that the enzyme was incapable of reducing D-mannuronic acid to D-mannonic acid. This is in marked contrast to D-glucurono-γ-lactone and D-galacturonic acid methyl ester which definitely are converted into L-ascorbic acid. The only difference between D-mannuronic acid and D-glucuronic acid is in the configuration of the groups on carbon atom 2.

The discovery that the conversion of D-glucose to L-ascorbic acid probably proceeds along a pathway similar to that described in scheme 5 raises the important question as to what difference there is between animals such as guinea pigs and man,

which can suffer from scurvy and are incapable of synthesizing L-ascorbic acid, and those, such as the rat, which do not suffer from scurvy. It appears that in all the animals the synthesis can proceed as far as D-glucuronic acid; even in man, normal urine contains 150–200 mg. of D-glucuronic acid per day, and when compounds such as menthol are administered the excretion of conjugated glucuronides is increased many times. The obstacle to the conversion of D-glucose into L-ascorbic acid would therefore appear to lie between D-glucuronic acid and L-ascorbic acid. In a later paper it is hoped to consider this question in more detail.

The discovery of a series of reactions which describe in broad outline the pathway by which the hexose sugars are converted into L-ascorbic acid is important because it opens up the subject for rapid advances on the enzymic side, though it is possible that the reactions involved in the transformation may not be so simple as in the above scheme. The experimental evidence does not allow us to determine the exact ring structure of the sugar derivatives which are the precursors of L-ascorbic acid, for in the solution in the cell fluids several different forms of the sugar derivatives will be present. In addition, no attention has yet been given to phosphorylated derivatives because at present there is no experimental evidence which definitely indicates that phosphate is concerned, but the intermediates in the transformation may well be phosphorylated analogues of the sugar acid compounds described above.

The fact that the ring structure of the γ-lactones of L-gulonic and L-galactonic acids is similar to that of L-ascorbic acid suggests that the intermediate may be a γ-lactone. However, the ready conversion of the methyl ester of D-galacturonic acid (no lactone ring is present) into L-ascorbic acid and the fact that the γ-lactones are formed very slowly at room temperature in the absence of catalysts suggests that the active intermediate may be a derivative of the sugar acid other than the lactone.

SUMMARY

1. Four compounds, L-gulono-, L-galactono- and D-glucurono-γ-lactones and D-galacturonic acid methyl ester, when fed to cress seedlings or injected into rats, have been shown to be transformed into L-ascorbic acid. A number of other sugar acid lactones with closely related configurations such as L-idono and L-talono-γ-lactones are inactive, except that two lactones, D-mannono-γ-lactone in the rat and D-altrono-γ-lactone in the cress seedling, are converted into D-*arabo*ascorbic acid.

2. The four compounds described above have been postulated as intermediates in a hypothetical series of reactions linking D-glucose (and in a similar series D-galactose) with L-ascorbic acid. The series of reactions in outline are:

D-glucose → D-glucuronic acid → L-gulonic acid → L-ascorbic acid

and

D-galactose → D-galacturonic acid → L-galactonic acid → L-ascorbic acid.

3. The specificity of the enzymes catalysing the transformation of the aldonic acid to L-ascorbic acid requires that the hydroxyls on carbon atoms 2 and 5 (compared to L-ascorbic acid) shall have the L-configuration and that on carbon atom 4 the D-configuration. An additional degree of specificity is implied by the fact that D-altrono-γ-lactone in cress seedlings and D-mannono-γ-lactone in the rat are converted into D-*arabo*ascorbic acid. This would suggest that the enzyme in cress seedlings will react only with compounds which have a configuration for the first four carbon atoms similar to L-galactonic acid (D-altronic acid) whereas the enzyme in the rat will react only with those which have a configuration similar to L-gulonic acid (D-mannonic acid).

4. A survey of a number of possible mechanisms by which the hexoses might be transformed into L-ascorbic acid has shown that those which involve L-glyceraldehyde, L-sorbose, D-sorbitol and D-gluconic acid are not present in cress seedlings or the rat.

We are indebted to Dr Dorothy M. Needham, F.R.S., of the School of Biochemistry, Cambridge, for the gift of a sample of D-glyceraldehyde. Mr F. C. Barrett has assisted with many of the preparations of the sugar lactones and the chromatographic isolation of D-glyceric acid. The work described in this communication forms part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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Isolation of D-Glyceric Acid from Cress Seedlings and its Relationship to the Synthesis of L-Ascorbic Acid

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In the preceding paper (Isherwood, Chen & Mapson, 1953) it has been shown that the transformation in cress seedlings and rats of the two common hexoses D-glucose and D-galactose into L-ascorbic acid can be represented in outline by the following series of reactions:

D-glucose → D-glucurono-γ-lactone → L-gulono-γ-lactone → L-ascorbic acid

and

D-galactose → D-galacturonic acid methyl ester → L-galactono-γ-lactone → L-ascorbic acid

The possibility that the transformation might occur as the result of the breakdown of the hexose into two three-carbon compounds and that the synthesis of L-ascorbic acid might start from these was also considered but was rejected in favour of the scheme given above in which the carbon chain of the hexose is not broken.

The present paper describes for the first time the isolation of the three-carbon compound, D-glyceric acid, from cress seedlings and examines the relationship between the synthesis of D-glyceric acid and the synthesis of L-ascorbic acid during the growth of the seedlings. D-Glyceric acid was shown not to be a precursor of L-ascorbic acid though its synthesis is in some way coupled with that of L-ascorbic acid.

EXPERIMENTAL

Methods

Culture of cress seedlings. This has been described previously (Isherwood *et al.* 1953). In most of the preliminary experiments the seedlings were grown in 0·02 M-$(NH_4)_2SO_4$ or water or 0·04 M-$NaHCO_3$ for 96 hr. It was found that the amounts of L-ascorbic acid present at this time in each group of seedlings were approximately 0·2, 0·6 and 0·9 mg./100 seedlings, respectively. After 96 hr. the rate of formation of L-ascorbic acid began to decline. If the seedlings are grown for shorter periods, the rate of L-ascorbic acid synthesis may be somewhat higher, but the seedlings are much smaller and it is difficult to separate the testas from them.

Estimation of L-ascorbic acid and sugars. The methods used have been described previously (Isherwood *et al.* 1953).

Chromatography. In preliminary experiments an extract of germinating cress seedlings, prepared by grinding them with an equal weight of ethanol and centrifuging, was examined in a paper chromatogram using either *n*-butanol:acetic acid:water (4:1:5 by vol. respectively) or

n-propanol: conc. NH_3 (7:3 by vol. respectively) as solvent. The apparatus and procedure were essentially the same as those described by Isherwood & Jermyn (1951) for the sugars. Amino acids were detected by spraying with 0·1% (w/v) ninhydrin in moist butanol and heating at 100° for 10 min. Sugars and acids were detected by spraying with 0·1 M-$AgNO_3$ in 4 N aqueous NH_3 and then heating at 100° for 20 min. Acids were also detected by spraying with 0·1% (w/v) thymol blue in water to which sufficient 0·1 N-NaOH had been added to render the solution deep blue. Phenols and phenolic acids were detected either by examination under ultraviolet light, which caused many of them to fluoresce, or by spraying with 1% (w/v) $FeCl_3$ in water or 8% (w/v) benzenediazosulphonic acid in water.

Examination and estimation of organic acids. The acids were extracted by the method described by Isherwood (1946) using a silica gel column and then separated and estimated by the paper chromatographic method described by Isherwood & Hanes (1953). The phenols and phenolic acids were removed by treating the extract of acids, before examination on a paper chromatogram, with a very slight excess of benzenediazonium sulphate (1% w/v in water) and then $Ba(OH)_2$ to remove the H_2SO_4 present in the solution. The precipitated azo compounds and $BaSO_4$ were removed by centrifuging. The presence of an excess of the diazo reagent was detected by using a solution of 2-naphthol in dilute alkali as an external indicator. The traces of azo compounds still in solution usually had very much higher R_F values than the organic acids or the original phenols, and did not interfere in the subsequent examination on the paper chromatogram. Usually 300 seedlings were taken for each analysis.

Experiments on rats. The rats were housed and fed as described in the previous paper (Isherwood *et al.* 1953). In the few experiments in which the rats were dosed with chloretone by mouth (20 mg. of the drug dissolved in five drops of arachis oil given to each rat every day by a dropping pipette), the urine was collected using toluene as a preservative (no oxalic acid was used). This urine was then examined chromatographically for the presence of compounds such as D-glyceric acid by the method of Isherwood & Hanes (1953).

Preparation of chemicals

D-*Glyceric acid.* This was prepared from a commercial sample of DL-glyceric acid by resolution with quinine (Anderson, 1909). The final material was a colourless syrup. Quinine salt m.p. 178°, $[\alpha]_D^{20} - 116°$ in water (*c*, 1). Calcium salt, $[\alpha]_D^{20} + 10·9°$ in water (*c*, 5). (Found: Ca, 14·0. $(C_3H_5O_4)_2Ca, 2H_2O$ requires Ca, 14·0%.)

D-*Glyceric acid ethyl ester.* This was prepared by heating 10 g. of the free acid in 50 ml. of absolute ethanol at 190° for 4–5 hr. in a sealed glass tube. The ethanolic solution was concentrated and the residual syrup distilled in a high vacuum. The clear, colourless, viscous liquid had $[\alpha]_D^{20}$ approx. + 7° in water (*c*, 10) and b.p. 110°/2–4 mm. The DL-ester was prepared in a similar manner.

L-*Gulono-γ-lactone.* This was prepared from D-glucurono-γ-lactone by reduction in slightly alkaline solution with sodium amalgam (Thierfelder, 1891). The lactone had m.p. 180° and $[\alpha]_D^{20} + 55°$ in water (*c*, 1).

D-*Glucurono-γ-lactone.* This was a commercial sample. The m.p. and rotation were identical with the figures given for the pure material.

RESULTS

Preliminary experiments

In a few experiments a crude extract of germinating cress seedlings which had been cultured in water was examined on a paper chromatogram. The assumption was made that the precursors would be at their highest level when synthesis was most active. Examination of the paper chromatograms showed the presence of a very large number of overlapping spots, none of which could be linked with the synthesis of L-ascorbic acid. These early experiments indicated that to obtain any useful information by this method, it was essential to be able to dissociate the synthesis of L-ascorbic acid from the normal growth of the seedlings. Comparative examination of cell saps from seedlings with a high and low rate of synthesis might then reveal which compounds were significant in the synthesis.

The experiments which were carried out subsequently were based on this general idea. The cress seedlings were cultured in different nutrient solutions to change the rate of synthesis of L-ascorbic acid and were then examined as before on a paper chromatogram. Mapson, Cruickshank & Chen (1949) had shown that culture in different nutrient solutions (0·02 M ammonium sulphate, water and 0·04 M sodium bicarbonate) changed the pH of the sap (4·2, 4·6 and 5·2, respectively) and that this influenced the synthesis of L-ascorbic acid (0·2, 0·6 and 0·9 mg./g. at 96 hr.) from the hexose sugars; detailed analysis of the results showed that the rate at which L-ascorbic acid was synthesized could be changed independently of the hexose content of the tissue. The seedlings in all cases grew normally, the respiration and appearance being very similar, so that the synthesis of L-ascorbic acid appeared to concern only a minor part of the metabolism. A preliminary experiment in which a careful comparative examination was made of the sap of seedlings grown as described by Mapson *et al.* (1949) showed that the acids present changed markedly with a change in pH. Sugars and amino acids did not change appreciably. Later experiments in which the organic acids were separated from the other substances present in the crude extract by using the silica gel column method of Isherwood (1946) and were then examined on a paper chromatogram showed that while the total amount of acid present changed with a change in the pH of the cell sap, not all the acids were similarly affected. One acid, later identified as D-glyceric, appeared to vary directly with the amount of L-ascorbic acid present in the original cress seedlings and later experiments were mainly concerned with this acid. Tracings of comparable chromatograms from seedlings grown in 0·02 M ammonium sulphate, water and 0·04 M sodium bicarbonate for 96 hr. are shown

in Fig. 1. The organic acids were extracted from the crude acidified extracts of the cress seedlings by the method of Isherwood (1946) and then examined on a paper chromatogram using *n*-propanol:concentrated ammonia as developing solvent.

It can be seen that the relative sizes of the glyceric acid spots are very roughly proportional to the above-mentioned amounts of L-ascorbic acid in the original seedlings. These paper chromatograms had been sprayed with ammoniacal silver nitrate and the depth of the staining is known to be only very roughly proportional to the amount of glyceric acid present, so that no great reliance can be placed on the accuracy of visual comparisons. However, the observation that there might be a link between glyceric acid and L-ascorbic acid was rendered more likely by a consideration of the behaviour of the other acids. In cress seedlings grown in ammonium sulphate and water the total amount of acid present in the cell sap is virtually the same (equivalent to 11·9 and 12·5 ml. of 0·1N alkali/300 seedlings after 96 hr.) and examination of extracts of the acids from the two groups indicates that, of all the acids present, only glyceric acid appears to change markedly. In seedlings grown in sodium bicarbonate the total amount of acid in the cell sap is larger (equivalent to 16·5 ml. 0·1N alkali/300 seedlings after 96 hr.) and an examination of an extract of the acids indicates that, while malic acid has increased noticeably and possibly accounts for a good deal of the increased acid, all the acids have increased. In spite of this general increase, the proportionality between the amounts of glyceric acid and L-ascorbic acid still persists and it appears that the relation between the two is largely independent of the mechanism regulating the other organic acids.

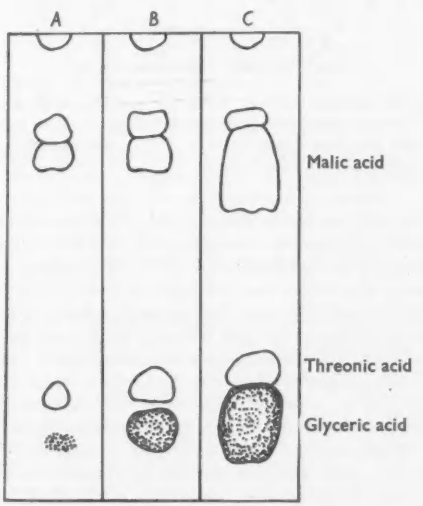

Fig. 1. Tracing of chromatogram of the acids from cress seedlings grown in 0·02M-$(NH_4)_2SO_4$ (*A*), water (*B*), and 0·04M-$NaHCO_3$ (*C*), at 20° in the dark. Paper, no. 1 Whatman. Solvent, *n*-propanol:conc. NH_3 (7:3 by vol.). Papers sprayed with ammoniacal $AgNO_3$ and heated at 100° for 20 min.

In certain experiments the possibility was considered that the glyceric acid might arise from L-ascorbic acid during the process of extraction. L-Ascorbic acid (10 mg.) was added to one-half of the extract from 300 cress seedlings (acidified with sulphuric acid, pH 2·0), the other half being used as a control. Both portions were then treated as described above and the organic acids examined on a paper chromatogram. Comparison of the chromatograms from the two portions showed that the very small spot due to L-threonic acid on the chromatogram of the control portion had been very greatly increased by the addition of the L-ascorbic acid, but that the spots due to glyceric acid were very similar on both chromatograms. The amount of L-ascorbic acid added was about 10 times the normal amount present in the seedlings. It seemed clear that the glyceric acid did not originate from the L-ascorbic acid during extraction.

Isolation of D-glyceric acid

Sufficient D-glyceric acid for a chemical examination was isolated by a large-scale repetition of the methods used for the qualitative examination of the acids (Isherwood & Hanes, 1953). The acids were finally separated on a paper chromatogram using *n*-propanol:concentrated ammonia as developing solvent. The appropriate pieces of paper containing the glyceric acid were extracted chromatographically with water and the extracts dried *in vacuo*. About 120 mg. of syrupy ammonium glycerate were obtained from 60 ml. of the original extract prepared from 3000 seedlings which had been grown for 96 hr. in water.

The chromatographic behaviour of the ammonium glycerate was the same as that of an authentic specimen of ammonium D-glycerate either alone or mixed together. *n*-Butanol:acetic acid:water and *n*-propanol:concentrated ammonia were used as solvents. The ammonium glycerate could readily be distinguished from the salts of either glycollic or threonic acid, substances which might be expected in an extract from plant material. On the paper chromatogram it gave a characteristic purple-black colour with the ammoniacal silver nitrate reagent, a colour which was also given by threonic acid but not by glycollic acid. The crude ammonium glycerate had $[\alpha]_D^{20}$ approx. $+10°$ (*c*, 0·24) in dilute ammonia, and $[\alpha]_D^{20} + 77°$ (*c*, 0·18) in 6% (w/v) ammonium molybdate. A sample of authentic

ammonium D-glycerate had $[\alpha]_D^{20} + 14°$ (*c*, 0·3) in dilute ammonia and $[\alpha]_D^{20} + 154°$ (*c*, 0·21) in 6% (w/v) ammonium molybdate. The figures for the rotation would suggest that the glyceric acid isolated was mainly D-glyceric acid but that some was present in the racemic form. This conclusion was confirmed by an examination of the calcium salt prepared by the method of Nef, Hedenburg & Glattfeld (1917). This slowly crystallized from aqueous ethanol but was extremely difficult to recrystallize. The impure calcium salt had $[\alpha]_D^{20} + 9°$ in water (*c*, 2·7) and $[\alpha]_D^{20} + 89°$ in 6% (w/v) ammonium molybdate (*c*, 2·1). For a sample of authentic calcium D-glycerate the figures were $+10·1°$ and $+107°$, respectively. The calcium content was 13·5; calc. for $(C_3H_5O_4)_2Ca, 2H_2O$, 14·0%.

Quantitative relationship between D-*glyceric acid and* L-*ascorbic acid in cress seedlings*

The evidence from a preliminary chromatographic examination of the cell sap of seedlings grown in 0·02M ammonium sulphate, water and 0·04M sodium bicarbonate for 96 hr. showed that the relative amounts of D-glyceric and L-ascorbic acids remained roughly the same in spite of culture in different nutrient solutions. The implication was that the formation of D-glyceric acid and the synthesis of L-ascorbic acid were linked together. This has been confirmed by later experiments in which the relation between the two acids was examined on a quantitative basis throughout the growth of the seedling (up to 9–10 days) and at different temperatures. The glyceric acid was measured by the chromatographic method of Isherwood & Hanes (1953) which does not distinguish between the D- and L-isomers. The figure given for glyceric acid therefore represents the total amount in the tissue and though some of it was undoubtedly in the racemic form, the bulk was probably the D form so that the figure for glyceric acid has been assumed to convey a reasonably accurate measure of the D-glyceric acid present. In Fig. 2 the amounts of D-glyceric and L-ascorbic acids are plotted against the time in days from the start of germination for seedlings grown in 0·02M ammonium sulphate, water and 0·04M sodium bicarbonate at 20°.

From the results it is clear that in each group of seedlings the amounts of D-glyceric and L-ascorbic acids present vary in a very similar manner throughout the period of growth. The mean figures for the ratio of D-glyceric acid to L-ascorbic acid in each group of seedlings, 2·2, 4·5 and 7·0, respectively, suggest that the link between the two acids is affected by the pH of the cell sap, 4·2, 4·6 and 5·2, respectively, and that the ratio increases as the sap becomes more alkaline.

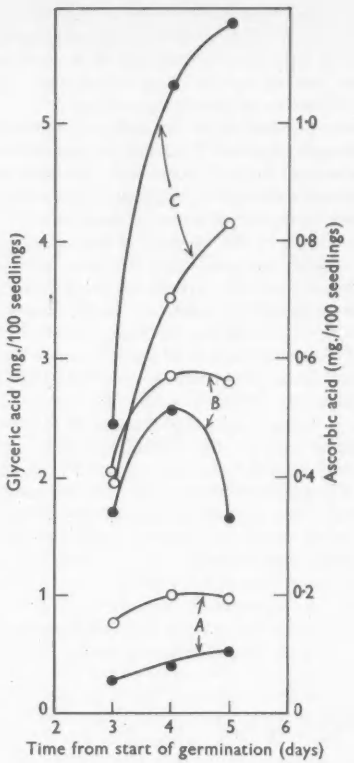

Fig. 2. Relation between D-glyceric and L-ascorbic acids in cress seedlings cultured in 0·02M-$(NH_4)_2SO_4$ (*A*), water (*B*), and 0·04M-$NaHCO_3$ (*C*), at 20°. ●—●, Glyceric acid; ○—○, L-ascorbic acid.

In addition to the effect of the pH of the cell sap on the synthesis of L-ascorbic acid, it is known that changing the temperature at which the seedlings are grown also affects the synthesis. It was therefore of interest to determine whether such a temperature change affected the link between the formation of D-glyceric and L-ascorbic acids; an experiment based on this is described in Fig. 3. The seedlings were cultured in water at 20°, some were left at this temperature and the others were transferred to a temperature of 1°. At 20° the synthesis of L-ascorbic acid proceeds normally, whereas at 1° it appears to be almost completely arrested. Transferring the seedlings back to 20° caused the synthesis to start again at much the same rate as before the change of temperature. The seedlings were analysed for sucrose, reducing sugars, L-ascorbic acid and D-glyceric acid. Sucrose and the reducing sugars were measured because they are known to be

affected by a change in temperature (Chen, 1950). The results show that lowering the temperature arrested both L-ascorbic and D-glyceric acids. The formation of the reducing sugars was partially arrested and the formation of sucrose was stimulated. Transfer back to 20° reversed the changes to the extent that L-ascorbic and D-glyceric acids were again formed, the reducing sugars rose rapidly and sucrose declined to a very low value; the formation of D-glyceric acid closely followed the synthesis of L-ascorbic acid in marked contrast to the behaviour of sucrose and the reducing sugars.

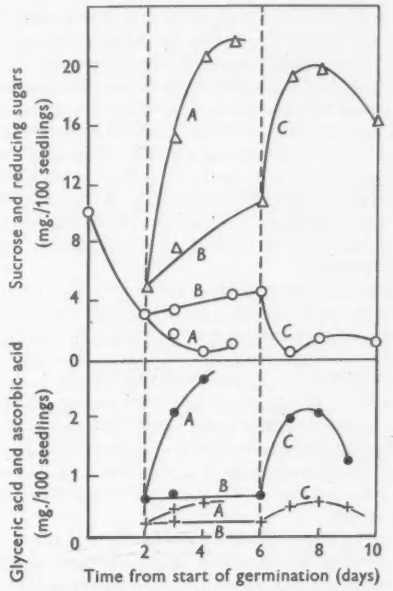

Fig. 3. Relation between D-glyceric acid, L-ascorbic acid, sucrose and reducing sugars in cress seedlings grown in water and subjected to changes of temperature. (*A*) Seedlings at 20°, (*B*) seedlings placed at 1°, (*C*) seedlings which had been at 1° changed to 20°. ○—○, Sucrose; △—△, reducing sugars; ●—●, glyceric acid; +—+, L-ascorbic acid.

Table 1. *Effect of feeding various sugar derivatives to cress seedlings on the formation of glyceric acid*

Substance fed (0·5%, w/v)	Duration of experiment (hr.)	Glyceric acid as % of control grown in water	L-Ascorbic acid as % of control grown in water
L-Ascorbic acid	96	76	630
D-Glucurono-γ-lactone	90	44	124
	114	83	120
	138	59	128
	162	77	150
L-Gulono-γ-lactone	72	12	154
	94	27	141
	120	34	144
	144	28	174

Another method of changing the synthesis of L-ascorbic acid in cress seedlings is to feed D-glucurono- and L-gulono-γ-lactones. These compounds have been shown (Isherwood *et al.* 1953) to increase the formation of L-ascorbic acid and, in the experiments now to be described, the effect on the relationship between glyceric and L-ascorbic acids was examined. The cress seedlings were cultured in 0·5% (w/v) solutions of L-ascorbic acid, and D-glucurono- and L-gulono-γ-lactones, and the formation of D-glyceric and L-ascorbic acids measured. The results are given in Table 1. The amounts of glyceric and L-ascorbic acids are expressed as a percentage of the corresponding amounts in seedlings grown in water. The results show that feeding L-ascorbic acid raised the L-ascorbic acid content of the seedlings until it was over 600% of the control but that the glyceric acid was only affected (80% of control) to a very small extent considering the large increase in L-ascorbic acid. Feeding D-glucurono- and L-gulono-γ-lactones also depressed the formation of glyceric acid (about 70% and 25% of the control value, respectively). The extra amounts of L-ascorbic acid present (30% and 50%, respectively) would seem to be too small to cause such an effect and the only reasonable explanation must be that the formation of D-glyceric acid is in some way coupled with the formation of L-ascorbic acid.

However, D-glyceric acid is not a precursor of L-ascorbic acid. Feeding an 0·5% (w/v) aqueous solution of ethyl DL-glycerate or D-glycerate (the ester was used as a convenient method of introducing glyceric acid into the cell; Beevers, Goldschmidt & Koffler, 1952) to cress seedlings did not increase significantly the formation of L-ascorbic acid, even though the glyceric acid in the sap increased by 20%.

Experiments on rats analogous to those on cress seedlings

The fact that there is a connexion between the synthesis of D-glyceric acid and the synthesis of L-ascorbic acid in the cress seedling made it of interest to determine whether any similar relationship existed in animals such as the rat which can synthesize L-ascorbic acid.

The rat is a convenient animal to study because other workers (Longenecker, Fricke & King, 1940; Smythe & King, 1942) have reported that an appreciable stimulation of the synthesis of L-ascorbic acid occurs as the result of the administration of chloretone, the excretion of L-ascorbic acid in the urine rising to several times the normal value. The excretion of other compounds also increases because

there is a marked increase in the excretion of conjugated glucuronides and if glyceric acid was formed in larger amount in the tissues, the excretion of this might rise also.

An experiment in which 20 mg. of chloretone were given to each rat every day showed that while the L-ascorbic acid in the urine rose from 3 in the case of the undosed controls to 20 mg./rat/day, the glyceric acid also rose from 2·6 to 4·6 mg./rat/day. This result suggests that there may be a connexion between the formation of the two compounds in the rat similar to that found in the cress seedling. To test whether D-glyceric acid is a precursor of L-ascorbic acid in the rat, 100 mg. each of ethyl DL- and D-glycerates were injected subcutaneously into rats, and the excretion of L-ascorbic acid in the urine was measured. No changes were observed and it was concluded that D-glyceric acid was not a precursor of L-ascorbic acid.

DISCUSSION

The isolation of D-glyceric acid from cress seedlings, in which it occurs in appreciable amounts under certain conditions (0·5 % of the fresh weight after the seedlings have been cultured for 5 days in 0·04M sodium bicarbonate solution), is interesting not only because the presence of this acid in plant materials has not been recorded previously, but also because it is a three-carbon compound and therefore may be closely connected with the breakdown of the hexoses in the seedlings. In the present study the main interest has been the relationship of the formation of this three-carbon compound to the formation of the six-carbon compound L-ascorbic acid in cress seedlings. Quantitative studies of the synthesis of the two compounds suggests that there is some connexion between them, for the formation of each runs parallel when the seedlings are cultured in different nutrient solutions and when they are subjected to rapid changes of temperature. The nature of the connexion is obscure for it is difficult to reconcile the previous observations with the fact that feeding precursors of L-ascorbic acid such as D-glucurono- and L-gulono-γ-lactones markedly depresses the formation of D-glyceric acid, though the very fact that the synthesis of D-glyceric acid is depressed supports the suggestion that there must be a connexion. In other plant tissues this connexion may be absent. Examination of mature strawberry leaves showed that they contained negligible amounts of glyceric acid ($<0·1$ mg./g.) yet the amount of L-ascorbic acid was several times that of cress seedlings. However, this result may be misleading if no synthesis of either compound was occurring in the leaves, for the connexion is assumed to be between the amounts undergoing synthesis and not between the total concentrations present.

Experiments on rats analogous to those on cress seedlings suggest that a similar connexion between the synthesis of D-glyceric acid and the synthesis of L-ascorbic acid also exists in the rat.

It is clear, however, that both in the cress seedling and in the rat, D-glyceric acid is not a precursor of L-ascorbic acid, since feeding the ester of D-glyceric acid to cress seedlings or injecting it into rats gave no increase in the synthesis of L-ascorbic acid.

The origin of the D-glyceric acid is unknown, but two suggestions have been tentatively considered.

(1) The D-glyceric acid is derived from 3-phospho-D-glyceraldehyde by dehydrogenation to 3-phospho-D-glyceric acid and loss of a phosphate group. In this connexion the work of Tewfik & Stumpf (1951) is suggestive. They found that the triose phosphate dehydrogenase only occurs in a limited number of tissues and is probably missing in leaf tissue so that the results obtained on strawberry leaves mentioned above could be explained by the absence of the dehydrogenase.

(2) The glyceric acid is produced by the oxidation of the glycerol, liberated as the fat in the seed breaks down during germination and growth. Rough calculations have shown that the amount of glycerol liberated is of the right order of magnitude. The main interest in this suggestion lies in the analogy between the oxidation of the primary alcohol group of the glycerol to give glyceric acid and that of the glucose (carbon atom 6) to give glucuronic acid as in the scheme described in the previous paper (Isherwood *et al.* 1953).

On the basis of either (1) or (2), the connexion between the synthesis of D-glyceric acid and the synthesis of L-ascorbic acid would lie in the fact that both require some common enzyme or coenzyme system.

SUMMARY

1. Cress seedlings cultured for 3–5 days in the dark at 20° were shown by chromatographic methods to contain from 0·05 to 0·5 % of D-glyceric acid/g. fresh weight. Quantitative studies of the amount of D-glyceric and L-ascorbic acids present throughout the period of growth of the seedling (up to 5 days) in various nutrient solutions (0·04M sodium bicarbonate, water and 0·02M ammonium sulphate) have shown that the formation of each runs roughly parallel. This is also true when seedlings cultured in water are subjected to changes in temperature during their growth.

2. Feeding D-glucurono- and L-gulono-γ-lactones, which are known to be precursors of L-ascorbic acid, to cress seedlings definitely depressed the formation of D-glyceric acid.

3. A somewhat similar relationship to that observed in the cress seedling between the formation

of D-glyceric and L-ascorbic acids possibly exists in the rat, for treatment with the drug chloretone causes the rat to excrete in its urine increased amounts of both D-glyceric and L-ascorbic acids.

4. It is clear, however, that D-glyceric acid is not a direct precursor of L-ascorbic acid, since feeding the ethyl ester of D-glyceric acid (the ester was used as a convenient method of introducing D-glyceric acid into the cell) to cress seedlings or injecting it into the rat did not increase the formation of L-ascorbic acid. The connexion between the synthesis of D-glyceric acid and the synthesis of L-ascorbic acid remains obscure.

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Biological Synthesis of L-Ascorbic Acid: the Conversion of L-Galactono-γ-lactone into L-Ascorbic Acid by Plant Mitochondria

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Evidence presented by Isherwood, Chen & Mapson (1953) has indicated that the synthesis of L-ascorbic acid *in vivo* in both plants and animals can be represented in outline by one or both of the following sequences:

(1) D-glucose→D-glucurono-γ-lactone→L-gulono-γ-lactone→L-ascorbic acid;

(2) D-galactose→D-galacturonic acid methyl ester→L-galactono-γ-lactone→L-ascorbic acid.

In the present paper an account is given of the enzymic conversion of L-galactono-γ-lactone to L-ascorbic acid by extracts of plant tissues. A following paper will deal with the conversion both of L-gulono-γ-lactone and L-galactono-γ-lactone to L-ascorbic acid by extracts from animal tissues.

EXPERIMENTAL

Selection of material. Most plant tissues contain highly active enzymic systems capable of oxidizing ascorbic acid, and this fact had to be taken into account before selecting a tissue in which to demonstrate the synthesis of ascorbic acid. Clearly it is more difficult to study the subject in tissues which are actively oxidizing the vitamin, the use of inhibitors of oxidases, e.g. cyanide, being precluded by the knowledge, from our earlier studies *in vivo*, that this also inhibits the synthesis (Isherwood, Chen & Mapson, unpublished observations). It was desirable to choose a tissue in which the rate of synthesis *in vivo* was known to be rapid and, for this reason, seeds during the early stage of germination were selected. Most of the experiments reported in this paper have been carried out with pea seeds, but sufficient work has been done with mung bean seeds to show that the pea seed is not exceptional.

Pea seedlings contain an active ascorbic acid oxidase, but fortunately for our purpose the oxidative enzyme activity in the very early stage of germination is low, not reaching its full development until some 120 hr. after germination at 25°. The varieties of pea seeds used included Laxton Superb, Kelvedon Wonder and Onward, and differed only in their degree of activity.

Enzyme material—plant tissues. Dry pea seeds were soaked in water for periods of 12–48 hr. at 20°. The soaked seeds (30 g.) were ground up with sand and 40 ml. of medium of the desired composition in a mortar. The brei was centrifuged at approximately 500 *g* for 5 min. and the supernatant solution removed. This solution is referred to in the text as the whole extract and cytoplasmic particles (mitochondria) were separated from it by centrifuging at 10000 *g* for 20 min. Washed mitochondria were prepared by suspending the residue from the first centrifugation in 20 ml. of the medium and recentrifuging, this procedure

being repeated several times. All of these operations were carried out at a temperature of 1°, the extraction apparatus and extraction media being cooled to this temperature before use. Unless otherwise stated, the tests for enzymic activity were carried out at 37°.

Estimation of ascorbic acid. This was carried out by removing 1 ml. samples, acidifying with 2% HPO_3, removing protein by centrifugation, and titrating ascorbic acid against 2:6-dichlorophenol indophenol.

L-*Galactono-γ-lactone.* This was normally added in a concentration of 0·5 mg. to 1 mg./ml. of reaction mixture. The lactone was prepared as described previously (Isherwood, Chen & Mapson, 1954), as also were the L-gulono-γ-lactone and the other sugar γ-lactones used in this work.

Oxygen consumption. This was estimated by the standard Warburg technique.

RESULTS

Conversion of L-*galactono-γ-lactone into* L-*ascorbic acid*

After many unsuccessful attempts, it was found that preparational procedures in which the tissue was extracted in the cold with a solution containing 0·4 M sucrose, 0·1 M phosphate and 4×10^{-3} M-Mg^{2+} were successful. These are identical with those used by Millerd, Bonner, Axelrod & Bandurski (1951) for the extraction of plant mitochondria. The extracts so obtained catalysed the formation of L-ascorbic acid from L-galactono-γ-lactone and the ascorbic acid so formed was identified as L-ascorbic acid by the chromatographic technique which we have already described (Chen, Isherwood & Mapson, 1953). The enzymic activity of these extracts was found to be centred in the mitochondrial elements of the cell (Fig. 1), there being little or no activity in the soluble fraction. These mitochondrial preparations did not catalyse the oxidation of ascorbic acid, hence the synthesis could be studied without the complications due to oxidase activity. This, however, as will be shown later, depends critically on the stage of germination.

The reaction proceeded at an almost linear rate, ceasing when about 40% of the lactone had been converted to ascorbic acid. The addition of a further quantity of the lactone at this stage led to the synthesis of further amounts of ascorbic acid, which indicates that the reaction stopped owing to the disappearance of substrate rather than to destruction of enzyme activity (Fig. 1).

Three possible explanations of the failure to obtain a higher percentage of conversion suggested themselves: (1) that under the experimental conditions a 40% conversion represented the equilibrium point of the reaction L-galactono-γ-lactone ⇌ L-ascorbic acid, (2) that galactonic acid inhibited the reaction and (3) that the lactone was converted into free acid. The first explanation was shown to be incorrect when it was found that L-ascorbic acid, added at the start of the reaction in a concentration

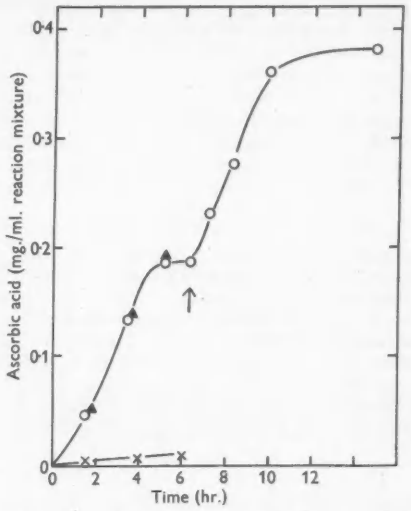

Fig. 1. The conversion of L-galactono-γ-lactone to L-ascorbic acid. Reaction mixture 0·4 M sucrose, 0·1 M-PO_4 (pH 7·4), 4×10^{-3} M-Mg^{2+}, containing initially 0·5 mg. L-galactono-γ-lactone/ml. ▲—▲, Whole extract (mitochondria + soluble fraction); ○—○, mitochondria; ×—×, soluble fraction of whole extract; ↑ denotes addition of a further amount of L-galactono-γ-lactone (0·5 mg./ml. reaction mixture).

equal to that synthesized in control experiments, had no effect on the rate of synthesis; the second was disproved by the fact that galactonic acid, added in equivalent amount to L-galactono-γ-lactone, had no influence on the subsequent course of the reaction. At a neutral pH the γ-lactones of the sugar acids are known to be converted slowly to the free acids and we have found that galactonic acid is not oxidized to L-ascorbic acid by these enzyme preparations. It appears probable therefore that the third explanation is correct and, if so, this would explain both the continual synthesis which occurs on addition of more of the lactone (Fig. 1), and the higher percentage conversion of the lactone to ascorbic acid as the rate of synthesis is increased. When the rate of the enzymic oxidation is high, as with mitochondrial preparations of great activity, a higher proportion of the lactone is changed to ascorbic acid than with preparations of lower activity. This is undoubtedly due to the fact that the longer reaction times involved in the second case permit a greater proportion of the lactone to be transformed into the non-reactive galactonic acid.

The mitochondrial preparations could be washed two to three times with the sucrose : phosphate : magnesium mixture without any decrease in the enzyme activity, although each successive washing reduced

the oxygen consumption of the mitochondria themselves. We have not as yet ascertained whether more exhaustive washing would have any influence on the activity of these preparations. Preliminary experiments indicate that the addition of the lactone increases the oxygen consumption of both the washed and unwashed mitochondria more than can be accounted for by the conversion of L-galactono-γ-lactone to L-ascorbic acid.

Conversion of related sugar-γ-lactones

In view of the previous work (Isherwood *et al.* 1954) in which both L-gulono-γ-lactone and L-galactono-γ-lactone were converted to L-ascorbic acid in intact plants and animals, we anticipated that preparations active in respect of L-galactono-γ-lactone would also catalyse the synthesis of L-ascorbic acid from L-gulono-γ-lactone. This we have not been able to demonstrate either with the whole extract or with mitochondrial preparations from pea and mung bean seeds; the latter extracts resemble those from pea seeds in their ability to synthesize L-ascorbic acid from L-galactono-γ-lactone. The reason for this remains obscure, especially, as we have found that L-gulono-γ-lactone fed to excised pea embryos increases the formation of L-ascorbic acid, though the reponse is much less than with L-galactono-γ-lactone. The relative formation of L-ascorbic acid in excised embryos grown in water, 0·5% L-gulono-γ-lactone, or 0·5% L-galactono-γ-lactone over a period of 24 hr. at 25° was 100, 175 and 1000, respectively.

The formation of D-*arabo*ascorbic acid only from D-altrono-γ-lactone in the plant, and only from D-manno-γ-lactone in the animal (Isherwood *et al.* 1953) prompted us to inquire whether this substance could be produced enzymically from these lactones and it was found that with the plant mitochondria, D-mannono-γ-lactone was not converted to L-ascorbic acid; on the other hand, D-altrono-γ-lactone was converted to D-*arabo*ascorbic acid.

L-*xylo*Hexulonic acid (2-keto-L-gulonic acid) has been suggested by some workers (Smythe & King, 1942; Smith, 1952) as a possible precursor of L-ascorbic acid, and Galli (1946) has claimed that *Aspergillus niger* can convert it to ascorbic acid: we failed however to detect any formation of L-ascorbic acid when L-*xylo*hexulonic acid was added to our enzyme preparations.

Rate of reaction as affected by concentration of L-galactono-γ-lactone and other related compounds

The maximum rate of conversion of L-galactono-γ-lactone to L-ascorbic acid occurs at low substrate concentrations. A substrate/velocity curve is shown in Fig. 2. The apparent Michaelis constant is of the order of 10^{-4} M. The overall rate of the reaction would appear to be limited by the rate of hydrogen transfer from the lactone rather than by the rate of oxygen activation by the cytochrome system for, as shown later, inhibitors which are known to reduce the activity of the latter had little or no effect on the rate of formation of ascorbic acid from the lactone.

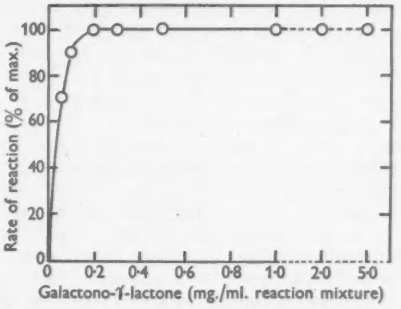

Fig. 2. The rate of formation of L-ascorbic acid as affected by substrate concentration.

Since we failed to find any evidence for the conversion of L-gulono-γ-lactone, or of D-manno-γ-lactone to ascorbic acid by the plant mitochondria, it was of interest to determine whether these substances exerted any competitive inhibition on the formation of L-ascorbic acid from L-galactono-γ-lactone.

Neither L-gulono-γ-lactone, D-galactono-γ-lactone, nor D-manno-γ-lactone when used in an equivalent concentration of 2 to 5 times that of L-galactono-γ-lactone, had any appreciable effect on the rate of conversion of the latter substance to L-ascorbic acid; also neither L-*xylo*hexulonic acid nor, as mentioned earlier, galactonic acid had any influence on the course of the reaction.

Isherwood *et al.* (1954) noted that DL-glyceraldehyde but not D-glyceraldehyde when fed to intact cress seedlings depressed the synthesis of L-ascorbic acid. In conformity with these results we have observed that DL-glyceraldehyde in concentrations of 10^{-3} M and higher inhibits the enzymic formation of L-ascorbic acid from the γ-lactone. On the other hand, we have also observed inhibition by D-glyceraldehyde. The different result obtained *in vivo* as against that obtained *in vitro* may be due to the more rapid metabolism of the D-isomer in *vivo*.

Effect of different extraction procedures

As previously mentioned, extraction of the tissue with sucrose in a concentration of 0·4 M with 0·1 M phosphate yielded the most active preparations. Very similar results on the activity of mitochondria in oxidizing acids of the tricarboxylic acid cycle have been reported by Millerd (1953), who found that the mitochondrial preparations possessed the greatest

oxidative activity when prepared with a sucrose: phosphate medium similar to that used by us. If phosphate was eliminated, the particles failed to oxidize α-oxoglutarate but could still oxidize succinate, whereas if extracted with phosphate alone they could still oxidise α-oxoglutarate. With pyruvate both sucrose, or mannitol, and phosphate were necessary, at all stages during the preparation.

The ability of mitochondria to convert L-galactono-γ-lactone to L-ascorbic acid appears likewise to be dependent on their full activity. If either sucrose or phosphate is omitted during extraction from the tissue, inactive preparations result, even though these substances are added subsequently to the extract. The effect on the activity of these mitochondrial preparations of altering the sucrose content of the media is shown in Fig. 3*a*. Mitochondria were extracted from peas with media containing 0·1 M phosphate and 4×10^{-3} M-Mg^{2+}, with different concentrations of sucrose. The extracted mitochondria were washed and resuspended in the same media for the reaction. The results show that a concentration of 0·4 M of sucrose appears to be optimum, with a fairly sharp decrease in activity at higher or lower levels of sucrose. Millerd found 0·3 M sucrose to be optimum for the oxidation of pyruvate by mung bean mitochondria.

We have confirmed the observations of Laties (1951) that sucrose may be replaced by mannitol, and have also extended this to glucose. It seems probable that many other non-electrolytes would behave similarly.

Influence of phosphate

Millerd believed that the necessity for having high phosphate levels in order to retain maximum activity was due to the inhibition of a phosphorolytic breakdown of some essential component. In support of this she found that 0·1 M fluoride, a phosphatase inhibitor, could replace the phosphate to a large extent. The rate of oxidation of α-oxoglutarate by mitochondria prepared by extraction with 0·4 M sucrose, 0·01 M phosphate and 0·1 M fluoride, was as great as when they were prepared by the higher phosphate: sucrose medium, but it is worth noting that the oxidation of pyruvate was, however, lower under these conditions. Our experiments have shown likewise that fluoride can only partially replace phosphate.

To determine the optimum amount of phosphate necessary mitochondria, prepared by extraction with 0·4 M sucrose, 0·1 M phosphate and 4×10^{-3}-Mg^{2+}, were washed once with a sucrose: magnesium medium containing different levels of phosphate. The washed mitochondria were then re-suspended in the same medium. The results of such experiments are illustrated in Fig. 3*b*; they show that reduction of phosphate below a level of 0·1 M seriously affects the

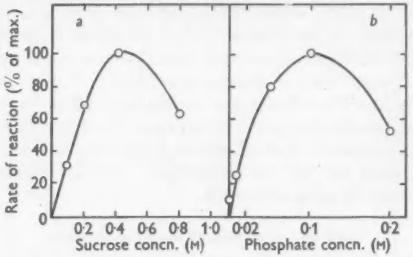

Fig. 3*a*. The influence of concentration of sucrose on the activity of mitochondria in converting L-galactono-γ-lactone to L-ascorbic acid.

Fig. 3*b*. The activity of mitochondria in converting L-galactono-γ-lactone to L-ascorbic acid, as influenced by the concentration of phosphate.

rate of the reaction. Such data give no indication as to whether phosphate is necessary for maintaining the integrity of the mitochondria, or as a participant in the reaction itself. Some evidence which indicates that the reaction is not dependent on the production of high-energy phosphate bonds is forthcoming from experiments in which it has been shown that mitochondrial preparations may be prepared by extraction with sucrose: arsenate mixtures, and washed 2 or 3 times with the same solutions, and yet possess as great an activity as when sucrose: phosphate mixtures are employed.

In view of such results, the influence of phosphate, as of arsenate, seems to us to be due to the effect these substances have in preserving and maintaining the integrity of the mitochondria. These results do not, of course, exclude the possibility that phosphate may be concerned in the oxidation of L-galactono-γ-lactone to L-ascorbic acid, though, so far, we have no direct evidence for this.

Influence of magnesium ions and adenosine triphosphate

Millerd (1953) showed in her experiments that both magnesium ions and adenosine triphosphate (ATP) when added to washed mitochondria caused a very considerable increase in the rate of oxidation of most of the acids of the tricarboxylic cycle. In contrast to this, the effect of magnesium ions on the oxidation of L-galactono-γ-lactone to L-ascorbic acid was small.

In these experiments, the mitochondria were prepared by extraction from the tissue with a magnesium-free sucrose: phosphate medium, washed once, and resuspended in the same medium. The omission of magnesium during extraction and washing reduced the rate by 10%, which was not increased if magnesium was subsequently added to the reaction mixture.

The addition of ATP in a concentration of 1×10^{-3}M to washed mitochondria cut down the rate of formation of ascorbic acid from galactono-γ-lactone by 40%. This inhibition was observed both in solutions of high (0·1M) and low (0·01M) phosphate concentrations and is not yet explained.

Effect of pH

The washed mitochondria were taken up in a sucrose:phosphate mixture containing 0·1M phosphate at different pH values and the results (Fig. 4*a*) show that the reaction is considerably affected by pH, the optimum value being in the region of 7·5 and falling sharply on either side. These results are similar to those reported by Slater & Cleland (1953) for the oxidation of α-oxoglutarate by cytoplasmic particles from heart muscle.

Hydrogen acceptors

As was to be expected, the oxidation of L-galactono-γ-lactone is dependent on oxygen and there is no reaction in its absence. Using washed mitochondria the oxygen tension may be reduced to 0·5% without any effect in the rate of formation of L-ascorbic acid (Fig. 4*b*).

Other substances have been tested to determine their ability to act as hydrogen acceptors. Under anaerobic conditions the rate of reduction of methylene blue by washed mitochondria was not increased by the addition of the lactone. Oxidized glutathione, dehydroascorbic acid and fumarate cannot function as hydrogen acceptors.

Action of cyanide, azide and carbon monoxide

The effects of cyanide, azide and carbon monoxide on the reaction were tested to determine whether the cytochrome system was involved. Cyanide (1×10^{-3}M) was found to reduce the rate of formation of ascorbic acid from the lactone very considerably, whether tested in the whole extract or on the mitochondria; with mitochondria alone an inhibition of 90% was obtained.

On the whole extract, azide ($1–4 \times 10^{-3}$M) inhibited the reaction almost completely during the first 2 hr. Subsequently, however, there was a considerable acceleration in the rate (Fig. 5*A*). When used in the same concentrations with mitochondria, azide inhibited the rate to only a small extent and in some experiments with azide at 1×10^{-3}M no inhibition was observed. This apparent anomaly was resolved when it was found that, with washed mitochondria, the inhibitory effect of azide was increased in the presence of respirable substrates, e.g. succinate or α-oxoglutarate (Fig. 5*B*, *C*). Evidently the residual cytochrome oxidase activity, left after treatment with azide, was sufficient to allow both the endogenous respiration of the mitochondria and any increase due to the addition of the galactono-γ-lactone to proceed at a maximum rate. In the presence of added substrates, competition for the available cytochrome was large enough in the case of succinate to suppress completely for a time, and in the case of α-oxoglutarate to reduce, the rate of formation of ascorbic acid from the lactone. As seen in Fig. 5*C*, with succinate the inhibition was only temporary but could be re-established by the addition of further succinate. In the absence of azide, in air, the addition of succinate at the levels tested had no adverse effect on the formation of ascorbic acid.

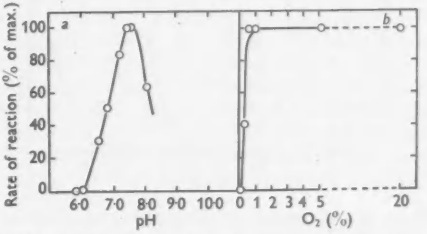

Fig. 4*a*. The influence of pH on the enzymic conversion of L-galactono-γ-lactone to L-ascorbic acid by mitochondria.

Fig. 4*b*. Effect of oxygen tension on the rate of formation of L-ascorbic acid from L-galactono-γ-lactone by mitochondria.

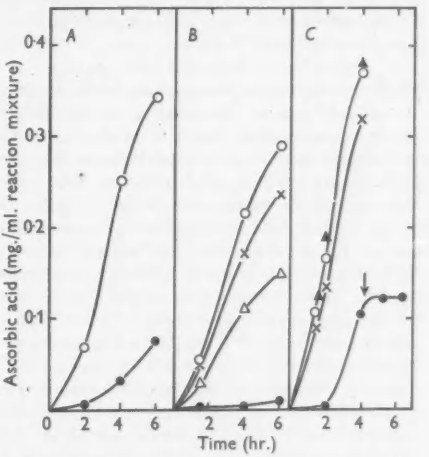

Fig. 5. The inhibitory effect of azide on the formation of L-ascorbic acid from L-galactono-γ-lactone on whole extracts (mitochondria + soluble fraction) and on washed mitochondria in the presence and absence of respirable substrates. (*A*) Whole extract: ○—○, control; ●—●, 4×10^{-3}M azide. (*B*) Mitochondria: ○—○, control; ×—×, 4×10^{-3}M azide; △—△, azide + 2×10^{-2}M α-oxoglutarate; ●—● azide + $2{\cdot}5 \times 10^{-2}$M succinate. (*C*) Mitochondria: ○—○, control; ▲—▲, 1×10^{-2}M succinate; ×—×, 4×10^{-3}M azide; ●—●, azide + 1×10^{-2}M succinate; ↓ addition of more 1×10^{-2}M succinate.

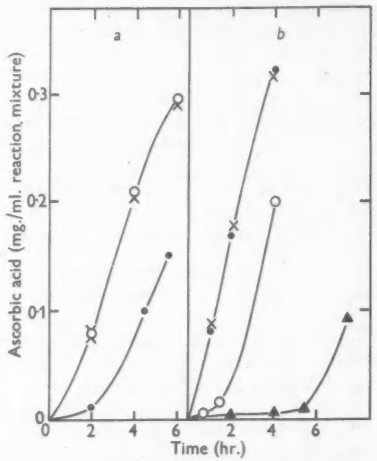

Fig. 6. The influence of CO on the formation of L-ascorbic acid from L-galactono-γ-lactone on whole extracts (mitochondria + soluble fraction) and on washed mitochondria in the presence and absence of succinate. All experiments carried out in the dark. (*a*) Whole extract: ○—○, in air; ×—×, in 95% N_2, 5% O_2; ●—●, in 95% CO, 5% O_2. (*b*) Mitochondria: ×—×, in 95% N_2, 5% O_2; ●—●, in 95% CO, 5% O_2; ○—○, in 95% N_2, 5% O_2 + succinate 2×10^{-2}M; ▲—▲, in 95% CO, 5% O_2 + succinate 2×10^{-2}M.

Very similar results were observed with carbon monoxide. With gas mixtures containing 95% CO and 5% O_2 in the dark, there was no inhibition of the reaction with washed mitochondria, but a temporary inhibition with whole extracts (Fig. 6*a*, *b*) when compared with controls containing 95% N_2, 5% O_2. As in the azide experiments, inhibition of the reaction due to the presence of carbon monoxide could be observed on the addition of succinate (Fig. 6*b*). A short temporary inhibition due to the addition of succinate was observed even with the controls in 95% N_2, 5% O_2, but a much longer one was observed with 95% CO, 5% O_2.

To demonstrate inhibition by carbon monoxide in mitochondria in the absence of added substrates, gas mixtures of 97·5% CO, 2·5% O_2 were made up. With these, an inhibition by carbon monoxide was observed, as compared with controls in gas mixtures containing 95% N_2, 2·5% O_2. This inhibition by carbon monoxide was reversed by light (Fig. 7).

The results described in this section indicate that the cytochrome system is involved as the terminal oxidase in the formation of L-ascorbic acid from L-galactono-γ-lactone. It seems clear also that, with mitochondria, the rate of formation of ascorbic acid is not limited by the activity of the cytochrome system.

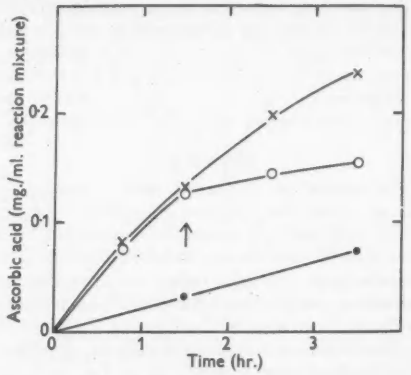

Fig. 7. Inhibition of the formation of L-ascorbic acid from L-galactono-γ-lactone by CO, and reversal by light. ×—×, Mitochondria in atmosphere of 97·5% N_2, 2·5% O_2; ○—○, mitochondria in atmosphere of 97·5% CO, 2·5% O_2 in light; ↑ denotes change from light to dark; ●—●, mitochondria in atmosphere of 97·5% CO, 2·5% O_2 in dark.

Effect of other enzyme inhibitors

We thought it of interest to examine the effect of 2:4-dinitrophenol (DNP) on the conversion of L-galactono-γ-lactone to L-ascorbic acid, because Loomis & Lippman (1948) have shown that this substance suppresses mitochondrial phosphorylative reactions without affecting those concerned with oxidation. Bonner & Millerd (1953) have further shown that 10^{-4}M DNP completely suppresses oxidative phosphorylation with plant mitochondria, without affecting oxygen consumption. The experiments we have carried out show that DNP (5×10^{-4} to 1×10^{-3}M) has absolutely no effect on the rate of conversion of L-galactono-γ-lactone to L-ascorbic acid. The same result is obtained whether the mitochondria have been washed or not. This evidence taken in conjunction with that given earlier in this paper suggests that phosphorylation reactions are not involved in the formation of L-ascorbic acid from L-galactono-γ-lactone.

Of the other inhibitors studied, fluoride (1×10^{-3} to 1×10^{-2}M) had no influence on the course of the reaction, whereas iodoacetate, arsenite and alloxan (each 1×10^{-3}M) inhibited the reaction by 30, 5 and 25%, respectively.

Activity of mitochondria prepared from peas at different stages of germination

Washed mitochondria were prepared from pea seeds after soaking for 17 hr. at 20°, and from seeds which had been germinated for periods varying from 1 to 5 days at 25°. Their activity in converting L-galactono-γ-lactone to L-ascorbic acid and in oxi-

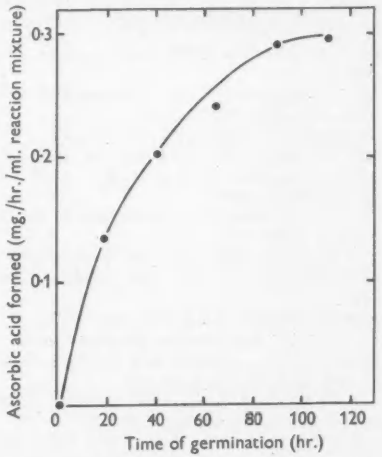

Fig. 8. Increase in enzymic activity of mitochondria prepared from peas at successive stages in germination. Washed mitochondria prepared from 5 g. of germinated peas suspended in 5 ml. of reaction mixture containing 0·4 M sucrose, 0·1 M phosphate (pH 7·4), and 4×10^{-3} M-Mg^{2+} with L-galactono-γ-lactone (1 mg./ml. reaction mixture).

dizing ascorbic acid was tested. The results (Fig. 8) show that extracts from ungerminated dry seeds did not synthesize ascorbic acid from the lactone but, on soaking, a very rapid increase of activity occurred and continued during the first few days of germination. It is worth noting here that this rate of increase of enzymic activity bears a striking resemblance to the rate of formation of ascorbic acid by intact pea seeds during germination, cf. Harris & Ray (1933). The ability of the mitochondria to oxidize ascorbic acid also increased during germination to such an extent that by the 4th to 5th day the initial rapid synthesis of ascorbic acid, which followed on the addition of galactono-γ-lactone to mitochondria prepared from such material, gave way to a very rapid oxidation of the ascorbic acid formed. The factors which govern the oxidation of ascorbic acid by mitochondria seem well worth further investigation. We have observed that unwashed mitochondria, prepared from pea seeds during the first 2 days of germination, do not catalyse the oxidation of added ascorbic acid but, on washing, the activity is noticeably increased. This problem requires further investigation.

DISCUSSION

The results presented in this paper show that cytoplasmic particles can, in the absence of added cofactors, oxidize L-galactono-γ-lactone to L-ascorbic acid. Our failure to observe a similar oxidation of L-gulono-γ-lactone suggests that the natural path of synthesis is via the galacturono-galactono-γ-lactone route rather than by that of glucurono-gulono-γ-lactone. Even more suggestive are the facts which will be presented in a later paper that D-galacturonic methyl ester is, and D-glucurono-γ-lactone is not, converted to L-ascorbic acid.

These observations do not however exclude the possibility that L-ascorbic acid may be synthesized by the gulono route, for the following reasons. The conversion of both glucurono-γ-lactone and L-gulono-γ-lactone to ascorbic acid by the intact seed during germination has been observed by us (Isherwood *et al.* 1954) for cress seedlings and we have found that this is also the case for pea seeds. It is true that the magnitude of the conversion of L-galactono-γ-lactone to ascorbic acid by the intact seedling is considerably greater than with L-gulono-γ-lactone, but this fact is susceptible to different interpretations.

Our failure to observe any synthesis of L-ascorbic acid from L-gulono-γ-lactone by mitochondrial preparations suggests that damage has been caused to some enzymic system necessary for the conversion. This presupposes either that there is a separate and more labile enzyme for the conversion of L-gulono-γ-lactone from that catalysing the corresponding conversion of L-galactono-γ-lactone, or, which seems more probable, that the route of synthesis is L-gulono-γ-lactone→L-galactono-γ-lactone→L-ascorbic acid, and that the ability of the mitochondria to effect the change L-gulono-γ-lactone to L-galactono-γ-lactone has been impaired.

An alternative explanation that L-gulono-γ-lactone may be metabolized along other lines besides being converted to L-ascorbic acid merits consideration, but seems unlikely in view of the fact that we have observed no trace of ascorbic acid being formed on addition of this lactone.

SUMMARY

1. The enzymic conversion of L-galactono-γ-lactone to L-ascorbic acid by mitochondria from pea and mung bean seeds has been demonstrated. Neither L-gulono-γ-lactone nor D-mannono-γ-lactone yield ascorbic acid in these circumstances.

2. Galactonic acid is not converted to L-ascorbic acid by these mitochondrial preparations, and the concurrent conversion of L-galactono-γ-lactone to galactonic acid during the reaction accounts for our failure to obtain quantitative yields of ascorbic acid from the lactone.

3. The conversion appears to be dependent on the integrity of the mitochondria. The most active preparations have been obtained by extraction with sucrose (0·4 M) and phosphate (0·1 M). Arsenate can

replace phosphate both in the extraction of the mitochondria and in the reaction medium.

4. The reaction is dependent on the presence of oxygen, but this may be reduced to a tension of 0·5% without any effect on the reaction velocity. Experiments with cyanide, azide and carbon monoxide suggest that the cytochrome system is involved.

5. 2:4-Dinitrophenol in high concentrations does not, but adenosine triphosphate (1×10^{-3} M) does inhibit the reaction. There is no evidence to suggest the necessity for the participation of compounds containing high-energy phosphate bonds in the formation of ascorbic acid from galactono-γ-lactone.

6. The enzymic activity of mitochondria prepared from peas increased rapidly in the early stages of germination.

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Oxidative Phosphorylation Coupled with the Oxidation of α-Ketoglutarate by Heart-muscle Sarcosomes

2. PHOSPHORUS:OXYGEN RATIO

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Ochoa (1944) was the first to show that the single step oxidation of α-ketoglutarate to succinate in dialysed heart extracts was accompanied by the esterification of inorganic phosphate. The number of atoms of phosphorus esterified per atom of oxygen consumed (the P:O ratio) in these experiments was 1·5, the same as obtained with pyruvate as substrate under otherwise identical conditions. Previously, Ochoa (1943) had shown that after correction for the heavy losses of esterified phosphorus due to the considerable adenosine triphosphatase and low hexokinase activities of his preparations, the P:O ratio with pyruvate was 3. Accordingly, Ochoa (1944) concluded that the corrected ratio with α-ketoglutarate as substrate was also 3.

In more recent work, the phosphorylation losses have been decreased by the addition of hexokinase and glucose. In this way Cross, Taggart, Covo & Green (1949) obtained a mean uncorrected value of $2{\cdot}43 \pm 0{\cdot}42$ (standard deviation) for 62 experiments with rabbit-kidney or rabbit-liver 'cyclophorase' preparations, while Hunter & Hixon (1949) obtained values, as amended by Hunter (1951), a little below 3 with a liver preparation. These determinations, as well as that given by Slater (1950) for a single determination with cat heart (2·79), agreed with Ochoa's conclusions.

In recent years, however, a number of workers have obtained ratios above 3. These, together with the earlier values, are summarized in Table 1. Values obtained with and without malonate have been listed separately. Unless malonate is used there will be some oxidation of the succinate formed from the α-ketoglutarate, resulting in somewhat lower ratios, since the P:O ratio for the oxidation of succinate to fumarate is lower than that obtained in the oxidation of other substrates. As a result of these recent findings and also on the basis of indirect arguments which will be considered in the Discussion, it is now widely believed that the true P:O ratio for the oxidation of α-ketoglutarate to succinate is 4 (e.g. see Hunter, 1951; Ochoa & Stern, 1952; Krebs, Ruffo, Johnson, Eggleston & Hems, 1953). Ochoa's (1943) original data are probably not sufficiently precise to distinguish between a P:O ratio of 3 and 4.

Table 1. P:O *ratios in oxidation of* α*-ketoglutarate*

Reference	Source of enzyme	Preparation	P:O (mean) Without malonate	P:O (mean) With malonate
Ochoa (1944)	Cat heart	Dialysed extract	—	3·0*
Cross *et al.* (1949)	Rabbit kidney and liver	'Cyclophorase'	2·4	—
Hunter & Hixon (1949)†	Rat liver	Washed particles	> 3	—
Slater (1950)	Cat heart	Sarcosomes (mitochondria)	2·79	—
Judah (1951)	Rat or rabbit liver or kidney	Mitochondria	3·4	—
Kielley & Kielley (1951)	Mouse liver	Mitochondria	2·6	3·3
Barkulis & Lehninger (1951)	Rat liver	Mitochondria	—	2·9
Copenhaver & Lardy (1952)	Rat liver	Mitochondria	—	3·6
Feldott & Plaut (1953)	Guinea pig and rat heart	Mitochondria	—	3·4
Krebs *et al.* (1953)	Rat liver	Dispersion of whole tissue	3·1	—

* Corrected for dephosphorylation losses. † Amended by Hunter (1951).

In earlier papers a number of factors which might affect the yield of phosphorylation associated with the oxidation of α-ketoglutarate to succinate by heart-muscle sarcosomes was studied (Slater, 1953*a*, *b*; Cleland & Slater, 1953; Slater & Cleland, 1953*a*, *b*; Slater & Holton, 1953). The following conclusions relevant to the subject of the present paper were drawn:

(1) *Measurement of phosphorylation.* Provided sufficient hexokinase is used, the amount of phosphate esterified is correctly measured.

(2) *Measurement of* P:O *ratio.* When ethylenediaminetetraacetic acid is included in the reaction mixture (Slater & Cleland, 1952), the P:O ratio is constant over a period of 45–60 min. at 25°. The oxidation proceeds at a uniform rate from the moment the sarcosomes are introduced into the reaction mixture (except for a slight fall towards the end of the period), so that the oxygen uptake can be accurately measured.

(3) *Isolation of sarcosomes.* The most active sarcosomes are obtained by isolation with isotonic media; saline and sucrose give preparations with the same P:O ratio.

(4) *Composition of reaction mixture.* The following variations of the reaction mixture had no appreciable effect on the P:O ratio, although the rate of oxygen uptake was often markedly affected: (i) pH between 6·2 and 7·7; (ii) tonicity between about half-isotonic and slightly hypertonic; (iii) phosphate concentration between 0·02 and 0·06 M; (iv) omission of cytochrome *c*; (v) concentration of adenosine diphosphate (ADP) between 10^{-4} M and $1{\cdot}2 \times 10^{-3}$ M; (vi) addition of adenosine monophosphate (AMP).

The reaction media used in investigations of oxidative phosphorylation contain a number of components, not all of which have been varied in the above-mentioned papers. This paper fills that gap and also considers other factors which could conceivably affect the P:O ratio. Having defined the conditions under which heart-muscle sarcosomes give the highest P:O ratio, all our experiments in this and related investigations which satisfy these conditions are assembled in order to estimate the most probable value of the P:O ratio. Since the conditions for the optimum P:O ratio in our experiments are not critical, but in every case cover a wide range of concentrations or treatment, it is considered likely that the results obtained can be extrapolated to the conditions of the intact heart. Our findings disagree with the recent assessments, and support Ochoa's original conclusion that the P:O ratio is 3.

METHODS

Sarcosomal preparations. These were made according to the procedure of Cleland & Slater (1953), using isotonic suspending media. Different types of preparations are designated according to the isolation medium and the animal used.

Measurement of activity of α*-ketoglutaric oxidase system and yield of oxidative phosphorylation.* Except where stated otherwise, the reaction mixture was that listed under 'standard conditions' in Table 10 of this paper or departed only slightly from these standard conditions. The reaction volume was 1 ml. in manometer flasks of 5–7 ml. total volume. Except in one experiment (Fig. 3), the reaction temperature was 25°. The experimental procedure has been described by Slater & Holton (1953).

The yield of oxidative phosphorylation is expressed either as P:O (number of atoms of inorganic phosphorus esterified per atom of oxygen consumed) or as P:α-Kg (number of atoms of inorganic phosphorus esterified per molecule of α-ketoglutarate disappearing). The number of atoms of inorganic phosphorus esterified was determined either as Δ'HMP (hexose monophosphate) which equals Δ HMP minus the myokinase control determined as described by Slater (1953*b*), or as Δ HMP plus Δ~P (~P = energy-rich reactive phosphate groups), as described by Slater & Holton (1953). The symbol Δ is used to denote the change in the amount of the component in the system since zero time.

The activity of the α-ketoglutaric oxidase system is expressed as Q_{O_2} (μl. O_2/mg. protein/hr.). One unit of the α-ketoglutaric oxidase system gives an oxygen uptake of 1 μl./hr.

RESULTS

Further investigations of method of preparation of sarcosomes

The method used for isolating the sarcosomes liberates only about 50–70 % of the total sarcosomes in the heart muscle; a considerable proportion remains trapped in the fragments of myofibrils separated from the dispersed tissue by a low-speed centrifugation. Cleland & Slater (1953) showed that these retained sarcosomes possessed a higher activity of the α-ketoglutaric oxidase system relative to that of cytochrome oxidase than the free sarcosomes. It was important, therefore, to determine whether they also gave a higher P:O ratio. This was investigated by measuring the P:O ratio of a dispersion of cat-heart muscle and of fractions isolated by differential centrifugation. Some combinations of fractions were also tried, in order to determine if the degree of separation of the fractions affected the P:O ratio. The supernatant obtained after sedimentation of the sarcosomes at 7500 *g* for 7·5 min. was recentrifuged at higher speed (14 300 *g* for 30 min.) in order to collect a fraction consisting of some sarcosomes and smaller granular material, possibly the microsomes (see Cleland & Slater, 1953). Although the P:O ratios obtained in this experiment (Table 2) are at the lower limit of the normal range of variation (see p. 35), it is clear that there is little difference between the ratios obtained with the dispersion and the three fractions. Certainly the sarcosomes retained in the residue had no greater phosphorylative activity than isolated sarcosomes (see also Table 3). Furthermore, addition of residue or of 'microsomes' to the sarcosomes did not affect the P:O ratio. In fact, the actual oxygen uptake and phosphorylation found with sarcosomes plus 'microsomes' were very close to that expected from the sum of the values obtained with the fractions separately.

The effect of varying the method of dispersing the heart was also studied. Two rat hearts were chopped coarsely and combined. About one-quarter of the total mince was dispersed in sucrose-ethylenediaminetetraacetic acid medium by treatment in a loosely fitting Potter & Elvehjem (1936) glass homogenizer. The remainder was ground with sand and the same medium in a glass mortar in the usual way and samples taken after 2, 3·5 and 6 min. grinding. The four samples were fractionated in the centrifuge as usual. The activities of the α-ketoglutaric oxidase system and the P:O ratios of each sarcosomal fraction and each residue from the low-speed centrifugation are summarized in Table 3.

Table 2. P:O *ratios of various fractions of cat heart muscle*

(For details of fractionation, see Table 3 of Cleland & Slater (1953). Dispersion = mixture immediately before first centrifugation. Residue = sediment after two centrifugations at 700 *g*. Sarcosomes = sediment after two centrifugations at 7500 *g*. 'Microsomes' = sediment after centrifugation at 14 300 *g*.)

Fraction	Q_{O_2}	P:O	P:α-Kg
Dispersion	11·6	2·04	2·64
Residue	6·9	2·01	—
Sarcosomes	70	2·27	2·93
'Microsomes'	19	2·17	3·06
Sarcosomes + residue	—	2·25	2·74
Sarcosomes + 'microsomes'	—	2·33	2·88

Table 3. *Effect of variations in the method of preparing dispersion of rat heart*

(See text for description of experiment. S = sarcosomes; R = sediment after two centrifugations at 700 *g*; Sup. = supernatant from centrifugation at 7500 *g*. In order to allow for the fact that the amounts of tissue in the different samples were not identical, the total enzyme activities have been calculated on the basis of a total protein content (determined by the sum of the protein contents of residue, sarcosomes and supernatant) of 100 mg.)

		Treatment			
		Ground with sand			Potter & Elvehjem homogenizer
		2 min.	3·5 min.	6 min.	
Protein (%)	S	8·0	11·6	12·2	9·4
	R	68	61	52	54
	Sup.	24	28	36	37
Activity of α-ketoglutaric oxidase system					
Q_{O_2}	S	83	67	50	57
	R	24	19	8	8
Total (units)	S	665	841	615	535
	R	1660	1140	420	450
	S + R	2325	1981	1035	985
P:O	S	2·74	2·64	2·55	2·47
	R	2·33	2·33	1·96	1·07
P:α-Kg	S	3·31	3·56	2·99	3·20
	R	2·96	2·98	2·70	—

As the time of grinding with sand was increased, the specific activity of the α-ketoglutaric oxidase system (Q_{O_2}) in both the sarcosomes and residue declined; the total enzymic activity also declined. The Potter & Elvehjem homogenizer gave fractions with activities similar to those obtained after 6 min. grinding with sand. The distribution of protein in the three fractions obtained using the Potter & Elvehjem homogenizer was also similar to that obtained after 6 min. grinding.

Despite the marked effects on the activity of the α-ketoglutaric oxidase system, the P:O ratio was only slightly affected by the degree of grinding, although there was a tendency for lower ratios with longer grinding. In most of our experiments, the time of grinding was 2 min., and it never exceeded 3·5 min. It is apparent that this procedure is somewhat milder than the use of a Potter & Elvehjem homogenizer. It should be mentioned that the tissue was given only a brief treatment in the Potter-Elvehjem homogenizer, less than that usually given in this method.

Stoicheiometry of the reaction

Relation between ΔO *and* Δα-Kg

Anaerobic reaction. For the accurate determination of the P:O ratio associated with the oxidation of α-ketoglutarate to succinate, it is necessary to remove side reactions as far as possible. As already briefly reported (Slater & Holton, 1952), crude preparations of yeast hexokinase catalyse an anaerobic reaction of α-ketoglutarate which is coupled with phosphorylation. The presence of this reaction is revealed when the disappearance of α-ketoglutarate (μmoles) exceeds the oxygen uptake (μatoms). This can be eliminated by using hexokinase of sufficient purity. We have found it necessary to proceed to step 5 of the procedure of Berger, Slein, Colowick & Cori (1946) to eliminate this reaction. Since the yield of phosphorylation in this side reaction is small (P:α-Kg = 0·5), errors in the estimated P:O ratio will become serious only when a considerable proportion of the α-ketoglutarate which reacts does so by this anaerobic reaction. This can be the case when large amounts of crude hexokinase are used, particularly with sarcosomes of relatively low oxidative activity, since the anaerobic reaction is relatively resistant to treatments which inactivate the α-ketoglutaric oxidase system.

Examples of the effect of this anaerobic reaction are given in Table 4. As will be shown presently, the mean value of the ratio of oxygen uptake (μatoms): α-ketoglutarate disappearance (μmoles) in the absence of this side reaction is 1·13. Assuming that the P:α-Kg of the anaerobic reaction is 0·5, it is possible to correct the observed P:O, as is shown in Table 4.

Effect of malonate and fluoride. Malonate is the most suitable reagent for the inhibition of the oxidation of the succinate formed from the α-ketoglutarate. However, the reaction mixtures used in studies of oxidative phosphorylation often contain fluoride which in the presence of phosphate is also a competitive inhibitor of this enzyme (Slater & Bonner, 1952). This is why malonate does not have as large an effect on the P:O ratio as might be expected (e.g. see Hunter & Hixon, 1949).

The effects of various concentrations of malonate and fluoride are shown in Tables 5 and 6. Table 5 shows that, in the absence of either of these inhibitors, 2 atoms of oxygen were consumed per molecule of α-ketoglutarate, i.e. the α-ketoglutarate was probably largely oxidized to fumarate. Either malonate (0·01 M) or fluoride (0·04 M) alone reduced this ratio to 1·2–1·3 and the two together brought it to near 1. Up to 0·01 M malonate had no inhibitory effect on the α-ketoglutaric oxidase system, measured by the disappearance of α-ketoglutarate, but a slight inhibitory action was apparent with higher concentrations.

Fluoride inhibited the α-ketoglutaric oxidase system, but had little effect on the P:O ratio. As with succinic dehydrogenase (Bonner, 1951), the inhibition by fluoride was greater at the higher concentrations of phosphate, but as the difference was more marked with 0·02 M fluoride than with 0·04 M fluoride, it seems likely that the effect of phosphate concentration is more compiicated than with succinic dehydrogenase (see Slater & Bonner, 1952). The inhibition of phosphorylation by 0·04 M fluoride in the presence of 0·015 M phosphate might be due to the removal of considerable amounts of phosphate in the form of magnesium fluorophosphate.

Table 4. *Effect of anaerobic reaction of α-ketoglutarate, catalysed by crude hexokinase preparations, on estimation of* P:O *ratio*

($\Delta'HMP_{corr.} = \Delta'HMP - 0{\cdot}5\,(-\Delta\alpha\text{-Kg} + \Delta O/1{\cdot}13)$; $P:O_{uncorr.} = \Delta'HMP/-\Delta O$; $P:O_{corr.} = \Delta'HMP_{corr.}/-\Delta O$.)

Expt.	$-\Delta O$ (μatoms)	$-\Delta\alpha$-Kg (μmoles)	Δ′HMP (μmoles)	$\Delta O:\Delta\alpha$-Kg	$P:O_{uncorr.}$	$P:O_{corr.}$
1	3·37	5·74	9·53	0·59	2·83	2·42
2	3·68	5·85	8·31	0·63	2·26	1·92
3	2·59	3·93	6·60	0·66	2·55	2·29

Table 5. *Effect of malonate, fluoride and pyrophosphate on the α-ketoglutaric oxidase system and on oxidative phosphorylation*

(Saline-ethylenediaminetetraacetic acid sarcosomes from rat heart.)

[Fluoride] (M)	[Malonate] (M)	[Pyrophosphate] (M)	$-\Delta O$ (μatoms)	$-\Delta\alpha$-Kg (μmoles)	$\Delta O:\Delta\alpha$-Kg	P:O	P:α-Kg
0	0	0	5·66	2·77	2·04	1·71	3·50
0	0·01	0	3·78	2·99	1·26	2·06	2·60
0·04	0	0	2·75	2·11	1·30	1·82	2·37
0·04	0·005	0	2·62	2·36	1·11	2·19	2·43
0·04	0·01	0	2·48	2·42	1·02	2·22	2·28
0·04	0·02	0	2·17	2·40	0·90	2·53	2·29
0·04	0·04	0	2·10	2·12	0·99	2·40	2·38
0	0	0·01	3·98	2·27	1·75	1·62	2·84
0·04	0	0·01	1·28	1·21	1·06	1·50	1·59

Table 6. *Effect of fluoride on the α-ketoglutaric oxidase system and on oxidative phosphorylation*

(Expts. 1 and 2, saline-ethylenediaminetetraacetic sarcosomes from rat heart; Expt. 3, isotonic-sucrose sarcosomes from rat heart.)

Expt.	[Phosphate] (M)	[Fluoride] (M)	$-\Delta O$ (μatoms)	$-\Delta\alpha$-Kg (μmoles)	Δ'HMP (μmoles)	P:O	P:α-Kg
1	0·036	0	3·55	3·66	8·42	2·38	2·32
	0·036	0·005	2·77	2·47	5·79	2·09	2·34
	0·036	0·01	2·24	2·17	5·60	2·50	2·58
	0·036	0·02	2·16	2·22	5·41	2·50	2·44
	0·036	0·04	1·88	1·84	4·53	2·42	2·46
	0·015	0	2·33	2·06	5·88	2·52	2·86
	0·015	0·02	1·83	1·69	4·12	2·26	2·44
2	0·04	0	6·29	—	16·5	2·62	—
	0·04	0·04	3·73	—	11·07	2·97	—
	0·02	0	6·15	—	15·5	2·53	—
	0·02	0·04	3·50	—	9·87	2·82	—
3	0·02	0	3·57	—	9·62	2·70	—
	0·03	0·04	2·24	—	5·68	2·54	—

Table 5 also compares the effect of pyrophosphate, another competitive inhibitor of succinic dehydrogenase (Leloir & Dixon, 1937; Keilin & Hartree, 1949; Tsou, 1951). This reagent was unsuitable, since it markedly inhibited both the α-ketoglutaric oxidase system and the phosphorylation, without appreciably affecting the proportion of the oxidation past succinate.

As a result of these experiments, it was decided to use 0·01M malonate and 0·04M fluoride for the measurements of the P:O ratio. Although fluoride had little effect on the P:O ratio, it was preferable to include it in order to inhibit the adenosine triphosphatase and myokinase (Slater, 1953*b*). These concentrations were not sufficient completely to inhibit the oxidation of succinate formed from the α-ketoglutarate. In twenty-one experiments, the ratio $\Delta O:\Delta\alpha$-Kg ranged from 0·95 to 1·28, with a mean of 1·13.

Fig. 1 shows the oxygen uptake with equivalent amounts of α-ketoglutarate and succinate under various conditions. In the absence of either malonate or fluoride, the oxygen uptake with α-ketoglutarate proceeded rapidly without any time lag until 2 atoms of oxygen per mole of α-ketoglutarate were consumed. It appears that the succinate is largely oxidized as soon as it is formed, so that its steady-state concentration must be quite small, in agreement with Green's cyclophorase concept. However, malate is rather slowly oxidized by heart-muscle sarcosomes and this is reflected in the slow rate of oxygen uptake after the consumption of 2 atoms of oxygen. The decline in the rate of oxygen uptake is not due to inactivation of the enzyme preparation which is stable for long periods in the presence of the reaction mixture and ethylenediaminetetraacetic acid (Slater & Cleland, 1952). In the presence of malonate and fluoride, α-ketoglutarate was rapidly oxidized until rather more than 1 atom of oxygen was consumed, after which the oxygen uptake continued at a low but appreciable rate, equal to that given with the same concentration of succinate under these conditions. By extrapolating this uniform rate to zero time, the oxygen uptake corresponding to the oxidation of α-ketoglutarate to succinate was obtained. This agreed closely with the calculated value, showing the absence of other side reactions.

Fig. 1 shows that the rate of oxygen uptake with succinate declines even before 1 atom of oxygen is consumed; this is very likely due to progressively increasing inhibition by oxaloacetate which, being a competitive inhibitor of succinic dehydrogenase, will inhibit to an increasing degree as the succinate concentration declines, even if the oxaloacetate concentration remains constant. The steadily decreasing activity of succinic dehydrogenase is probably the reason why no sharp break occurs in the rate of oxygen uptake with succinate, when 1 atom of oxygen has been consumed.

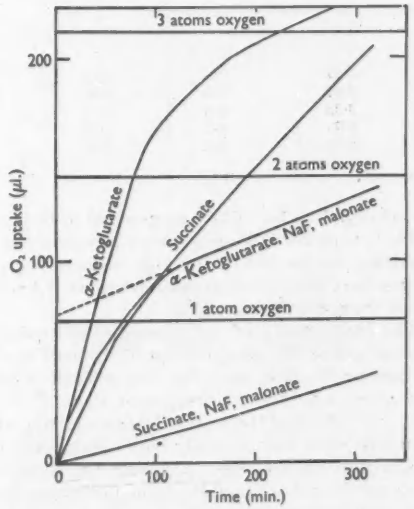

Fig. 1. Oxidation of 6·3 μmoles α-ketoglutarate or succinate by sarcosomes (sucrose-ethylenediaminetetraacetic acid preparation from rat heart) under various conditions. The horizontal lines show the O_2 uptakes corresponding to the consumption of 1, 2 and 3 atoms of oxygen per molecule of α-ketoglutarate or succinate. The reaction mixture contained 0·01 M malonate and 0·04 M fluoride, where indicated.

Table 7. *Comparison of uptake of inorganic phosphate with esterified phosphate*

(Sucrose-ethylenediaminetetraacetic acid sarcosomes from rat heart.)

Expt.	Δ′ HMP (μmoles)	−Δ Inorganic phosphate (μatoms)
1	4·1	4·2
	6·8	7·9
	9·4	10·9
	10·0	10·5
	10·6	10·5
	13·4	13·4
2	8·3	8·5
	8·2	8·2

Relationship between inorganic phosphate uptake and esterified phosphate

The following methods of measuring the yield of oxidative phosphorylation have been used: (1) measurement of Δ′HMP, which is ΔHMP minus the HMP synthesized by the non-oxidative phosphorylation of glucose by ADP, catalysed by myokinase and hexokinase (Slater, 1953*b*); (2) measurement of ΔHMP plus Δ∼P (Slater & Holton, 1953). The myokinase correction in method (1) is usually only a few per cent of ΔHMP and the two methods also agree within 1–2%. If inorganic phosphate is converted into a product not measured as HMP or ∼P (e.g. inorganic pyrophosphate), these methods will not measure all the uptake of inorganic phosphate. This possibility was tested by comparing our procedures with the more commonly employed measurement of the disappearance of inorganic phosphate. Table 7 shows that Δ′HMP agrees very closely with −Δ inorganic phosphate. Thus, under the conditions of our experiments, all the inorganic phosphate which disappears is accounted for by the esterified phosphate directly measured by the analytical procedures.

Effect of added magnesium

The effect of the concentration of added magnesium on the α-ketoglutaric oxidase system and on the P:O ratio is shown in Table 8. Since 0·002 M ethylenediaminetetraacetic acid was present in all experiments, and this combines firmly with an equivalent concentration of magnesium (Schwarzenbach & Ackermann, 1947), the concentration of free magnesium must have been very small when the total magnesium did not exceed 0·002 M. The concentrations of free magnesium in Table 8 have been calculated on the assumption that all the ethylenediaminetetraacetic acid is bound with magnesium. An insignificant proportion would have been combined with calcium or other metals in the sarcosomes or reagents.

It is apparent from Table 8 that the maximum rate of oxidation and of phosphorylation is obtained with very low concentrations of free magnesium and that the concentration can be varied widely on either side of the standard concentration (0·005 M total, 0·003 M free) without affecting the P:O ratio. Even in the presence of ethylenediaminetetraacetic acid, the rate of oxidation was considerable in the absence of added magnesium. This can be explained by Cleland's (1953) finding that sarcosomes contain large amounts of magnesium even when isolated in media containing ethylenediaminetetraacetic acid. The phosphorylation was more dependent upon the addition of magnesium, but this is undoubtedly because the added yeast hexokinase requires magnesium in solution. The apparent inhibition of

Table 8. *Effect of magnesium concentration on α-ketoglutaric oxidase system and on* P:O *ratio*

(The free Mg^{2+} is calculated on the assumption that all the ethylenediaminetetraacetic acid is bound with magnesium, but disregarding the possible formation of other magnesium compounds, e.g. magnesium fluorophosphate. The sarcosomes were prepared from rat heart in sucrose-ethylenediaminetetraacetic acid medium (Expt. 1), isotonic sucrose (Expt. 2) and saline-ethylenediaminetetraacetic acid (Expt. 3).)

Expt.	[Added Mg^{2+}] Total (m-moles/l.)	Free (m-moles/l.)	$-\Delta O$ (μatoms)	$-\Delta$α-Kg (μmoles)	Δ′HMP (μmoles)	P:O
1	0	0	—	—	0·3	—
	2·1	0·1	—	—	14·4	—
	5·3	3·3	5·67	—	15·3	2·70
	10·7	8·7	5·77	—	15·9	2·75
2	0	0	1·63	—	—	—
	2·1	0·1	2·08	—	—	—
	5·3	3·3	2·45	—	—	—
3	0	0	1·44	1·27	0·3	0·19
	1·0	0	2·84	2·67	4·6	1·62
	2·4	0·4	3·87	3·35	8·3	2·14
	10·7	8·7	4·09	3·71	9·7	2·38
	*21·3	19·3	7·94	3·50	9·8	1·23

* Copious ppt. observed.

Table 9. *Effect of α-ketoglutarate concentration on rate of oxygen uptake and on* P:O

(Saline-ethylenediaminetetraacetic acid sarcosomes from rat heart. The $[MgCl_2]$ used in this experiment was 2·4 m-moles/l. total, 0·4 m-moles/l. free.)

	[α-Ketoglutarate] (m-moles/l.) 2·35	4·69	7·0	14·0
Q_{O_2} (initial rate)	44·4	45·6	50·1	49·2
$-\Delta O$ (μatoms)	3·03	3·75	3·87	3·90
$-\Delta$ α-Kg (μmoles)	2·33	3·10	3·35	3·28
Δ O:Δ α-Kg	1·30	1·21	1·16	1·19
Δ′ HMP (μmoles)	6·4	8·1	8·3	8·1
P:O	2·10	2·16	2·14	2·07
P:α-Kg	2·74	2·60	2·48	2·46

Table 10. *Optimum conditions for measuring oxidative phosphorylation*

(Concentrations are m-moles/l. unless stated otherwise.)

	Optimum range	Standard conditions
pH	6·2–7·7	7·4
[Na or K phosphate]	20–60	30
[Na_2 or K_2 malonate]	5–40	10
[NaF or KF]	0–40	40
[$MgCl_2$]	3–10	5
[K_2 α-ketoglutarate]	2–14	5
[Glucose]	†	30
[AMP*]	0–1·2	0·6
[ADP*]	0·1–1·2	0·6
[Cytochrome *c*]	0–0·6	0·04
[KNa_2H ethylenediaminetetra-acetate]	0·1–2	2
[Osmolar] (M)	0·14–0·48	0·32
[Sarcosomes] (mg. protein/ml.)	0·12–1·25	0·8
Hexokinase (units/mg. sarcosomal protein)	>40	100

* K salts. † Sufficient to react with esterified P.

phosphorylation by 0·021 M magnesium in Expt. 3, Table 8, is probably largely due to precipitation of malonate and fluoride by this high concentration of magnesium, since $-\Delta O$ greatly exceeded $-\Delta$α-Kg under these conditions.

The high affinity of the α-ketoglutaric oxidase system and of the phosphorylating systems in the sarcosomes for magnesium shown in Table 8 does not agree with the conclusions of Paul, Fuld & Sperling (1952) and Harman & Feigelson (1952), who comment upon the unusually high requirement for magnesium of heart-muscle preparations. However, it is clear from the data of Paul *et al.* (1952) (see their Fig. 1) that magnesium did not activate the initial rate of oxidation, but stabilized the enzyme system. Such a stabilizing effect of magnesium, in the absence of ethylenediaminetetraacetic acid, has been reported by Slater & Cleland (1952).

Effect of concentration of α-ketoglutarate

The effect of varying the concentration of α-ketoglutarate on the rate of oxygen uptake and on the P:O ratio is shown in Table 9. It is clear that the α-ketoglutaric oxidase system has a high affinity for its substrate and that the P:O ratio is independent of the concentration of α-ketoglutarate over a wide range. The P:O ratios in the experiment shown in Table 9 are somewhat low, because a low concentration of magnesium chloride was used.

Optimum conditions for measuring P:O *ratio*

Results reported in this and in previous papers enable us to define the limits within which the components of the reaction mixture may be varied without appreciably affecting the P:O ratio of heart-muscle sarcosomes, with α-ketoglutarate as

Table 11. *Reproducibility of measurements of* P:O *and* P:α-Kg *ratios*

(Each measurement represents a single flask, each containing the same reaction mixture, the same amount of sarcosomes (sucrose-ethylenediaminetetraacetic acid preparation from rat heart) and the same number of hexokinase units. Eight different hexokinase preparations were used, varying from specific activity 174 to 750 (as defined by Berger *et al.* 1946).)

	1	2	3	4	5	6	7	8
$-\Delta O$ (μatoms)	3·03	3·03	3·06	3·13	3·10	3·16	3·24	3·22
$-\Delta\alpha$-Kg (μmoles)*	2·69	3·00	2·79	2·59	2·68	2·70	2·92	2·74
$\Delta O:\Delta\alpha$-Kg	1·13	1·01	1·10	1·21	1·16	1·17	1·11	1·17
Δ′HMP (μmoles)	7·99	8·34	8·24	8·60	8·40	8·41	8·70	8·75
P:O	2·64	2·75	2·69	2·75	2·71	2·66	2·69	2·72
P:α-Kg	2·97	2·78	2·96	3·32	3·14	3·11	2·98	3·19

* 7·69 μmoles initially present.

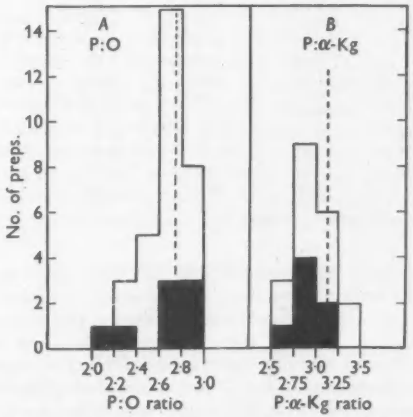

Fig. 2. *A*. Frequency distribution of P:O ratios obtained with thirty-two cat- and rat-heart sarcosomal preparations. Open, rat; blocked, cat. The dotted line shows the theoretical value, assuming P:O for the oxidation of α-ketoglutarate to succinate is 3, P:O for oxidation of succinate is 1, and $\Delta O:\Delta\alpha Kg = 1{\cdot}13$. *B*. Frequency distribution of P:α-Kg ratios obtained with twenty cat- and rat-heart sarcosomal preparations. Open, rat; blocked, cat. The dotted line shows the theoretical value, making the same assumptions as in *A*.

substrate. These are summarized in Table 10, which also includes the 'standard conditions' adopted in most of the experiments in this paper.

The reproducibility of a single measurement of the P:O ratio of a preparation is shown in Table 11. Eight different purified hexokinase preparations were used in this experiment which may account for the slight differences in oxygen uptake, since some hexokinase preparations contain an inhibitor of the α-ketoglutaric oxidase system. However, there is very little variation in the P:O ratio. Since the measurement of $\Delta\alpha$-Kg depends upon the difference between two separate analyses, it is subject to greater experimental error than ΔO. Thus, the reproducibility of P:α-Kg is not quite as good as that of the P:O.

The P:O *ratio for the oxidation of α-ketogluratate to succinate*

Fig. 2 describes all our measurements of P:O ratio and P:α-Kg ratio on cat and rat preparations which were carried out under the conditions defined by Table 10, using hexokinase of sufficient purity, and the procedure described by Slater & Holton (1953). No measurements have been omitted. Of the thirty-two sarcosome preparations, fourteen were made with saline medium, either with or without ethylenediaminetetraacetic acid, and eighteen were made with sucrose containing ethylenediaminetetraacetic acid. The average P:O ratio of the sucrose preparations was only slightly higher than the saline, but of the nine values below 2·6, six were for saline preparations and only three (out of eighteen) for sucrose preparations, including a value of 2·59.

Using the mean value of $\Delta O:\Delta\alpha$-Kg of 1·13 and assuming that the P:O ratio for the oxidation of succinate under the conditions of these experiments is 1·0 (Slater & Holton, unpublished experiments), it can be calculated that if the P:O ratio in the oxidation of α-ketoglutarate to succinate is 3, the observed P:O ratio would be 2·77 and the observed P:α-Kg would be 3·13. It is clear from Fig. 2 that the great majority of the observed P:O ratios are grouped very close to the theoretical value. The scatter of the P:α-Kg values is somewhat greater, probably for analytical reasons, but most of the values are only slightly below the theoretical value. The presence of a small amount of the anaerobic reaction of α-ketoglutarate would lower the P:α-Kg value somewhat without having any appreciable effect on the P:O ratio.

It is considered that the results of Fig. 2 point very strongly to a P:O ratio of 3 in the oxidation of α-ketoglutarate to succinate.

Investigation of Copenhaver & Lardy's method of measuring oxidative phosphorylation

Since our results do not agree with those of a number of other workers, it was necessary to compare our procedure with theirs. Copenhaver & Lardy's (1952) procedure, which is similar to that

Table 12. *Comparison of* P:O *ratios obtained with our reaction mixture and that of Copenhaver & Lardy* (1952)

(*Copenhaver & Lardy's reaction mixture.* Phosphate, 0·0135 M; malonate, 0·01 M; NaF, 0·01 M; ATP, 0·002 M; α-Kg, 0·0067 M; $MgSO_4$, 0·0074 M; cytochrome *c*, $1·2 \times 10^{-5}$ M in main flask. Glucose, 0·017 M; hexokinase, 70 units in side bulb. Sarcosomes (0·1 ml. of sucrose-ethylenediaminetetraacetic acid preparation from rat heart, containing 1·04 mg. protein) added to main flask at zero time, attached to manometer and placed in bath at 25°. Contents of side bulb tipped into main flask 3 min. later. Different flasks removed and contents immediately deproteinized 2 min., 17 min. and 27 min. after tipping. Δ refers to change in component from 2 min. after tipping.

Our procedure. Usual procedure with usual reaction mixture, same concentration of sarcosomes and hexokinase as above. 35 min. Results are shown for duplicate flasks.)

	Copenhaver & Lardy			Slater & Holton	
Time after tipping (min.)	2	17	27	—	—
$-\Delta O$ (μatoms)	—	1·96	2·99	4·09	4·26
α-Kg (μmoles)	5·71	4·21	3·28	1·16	1·26
$-\Delta$α-Kg (μmoles)	—	1·50	2·43	3·70*	3·60*
Δα-Kg:ΔO	—	—	1·23	1·10	1·19
∼P (μmoles)	1·98	1·37	1·19	0·68	0·69
Δ∼P (μmoles)	—	−0·61	−0·79	0·06*	0·07*
HMP	2·39	8·31	11·6	11·7	11·8
ΔHMP (μmoles)	—	5·92	9·2	11·7	11·8
ΔHMP + Δ∼P	—	5·31	8·4	11·7	11·8
P:O	—	2·72	2·82	2·86	2·76
P:α-Kg	—	—	3·46	3·16	3·26

* 4·86 μmoles α-Kg, 0·62 μmoles ∼P initially present.

used by a number of the authors listed in Table 1, was studied in detail.

Copenhaver & Lardy use a reaction mixture which does not differ in any important respect from ours, except for the omission of ethylenediaminetetraacetic acid, and the use of ATP as the adenine nucleotide. However, their procedure is considerably different. The reaction mixture containing all components except hexokinase and glucose is chilled to 0° in the main compartment of a Warburg flask containing two side bulbs. At zero time, the chilled mitochondrial preparation is added to the main compartment of the flask which is immediately attached to the manometer and placed in a bath at 30°. After 3 min. the hexokinase and glucose are tipped into the main flask from a side bulb; 2 min. later, that is 5 min. from adding the mitochondrial preparation, the manometer fluid is adjusted to zero, the taps closed and the oxygen uptake measured for a period of 15 min. The controls which serve as a basis for calculating the disappearance of inorganic phosphate are obtained by deproteinizing the contents of a flask 2 min. after tipping in the hexokinase and glucose.

This method was compared with our own in two types of experiments. The first was planned in order to determine if the reaction mixture and tipping procedure of Copenhaver & Lardy were themselves responsible for the higher P:O ratio. Consequently, their procedure was modified slightly in order to avoid certain technical difficulties inherent in the method which will be discussed below. The flask contents were at room temperature instead of at 0°, the bath temperature was 25° and differential manometers were used. In addition, ethylenediaminetetraacetic acid was included in the reaction mixture. None of these changes could lower the P:O ratio. Our method of extrapolating the oxygen uptake during the equilibration period was followed. Finally, the phosphorylation was determined by measuring the increase of esterified phosphate instead of the disappearance of inorganic phosphate. It has been shown above that the two methods of measuring the phosphorylation agree. It is clear from the results in Table 12 that Copenhaver & Lardy's procedure and ours give identical results.

In the second type of experiment, Copenhaver & Lardy's method of measuring the oxygen uptake was compared with ours, using the same preparations and our standard reaction mixture. Fig. 3*A*, curves 1*a* and 2*a* show the course of the manometric reading of duplicate flasks when Copenhaver & Lardy's procedure was followed exactly, except that water was tipped in at 3 min. instead of hexokinase and glucose which were already in the main flasks. There is apparently a considerable lag in reaching the maximum rate of oxygen uptake, although it has been shown with the identical reaction mixture and type of preparation that oxidation and phosphorylation proceed at a steady rate without any time lag (Slater & Holton, 1953). That the apparent lag is a manometric artifact is confirmed by the manometric record obtained with a control flask containing only water in the main compartment and treated in the same way as the experimental flask. It is clear that with the

apparatus used in this experiment, the procedure of Copenhaver & Lardy is inadequate to ensure temperature equilibration. When the curves 1*a* and 2*a* are corrected for the pressure changes in the control flask, the rates of oxygen uptake are practically identical with that obtained by our usual procedure, using differential manometers (curve 4, Fig. 3*B*). Curve 5, Fig. 3*B*, which like curve 3 represents the pressure changes in a control flask containing only water in the main compartment, shows that complete temperature equilibration is obtained in 10 min. with our procedure.

The magnitude of the error in measuring the oxygen uptake by direct calculation from the manometric readings, as in Copenhaver & Lardy's procedure, can be calculated from Fig. 3. The apparent oxygen uptake in 15 min. calculated in this way was 41·4 μl. (curve 1*a*) and 39·0 μl. (curve 2*a*) for the two flasks. The mean, 40·2 μl. (3·59 μatoms) is in the range of oxygen uptakes obtained by Copenhaver & Lardy (3·2–6·8, mean 4·7 μatoms). The actual oxygen uptake in 15 min. calculated from curve 4, allowing for the threefold difference in amounts of enzyme preparation and in volume, is 67·8 μl. = 6·04 μatoms. Thus the P:O ratio of 2·71 actually obtained by our procedure in this experiment would become $2{\cdot}71 \times 6{\cdot}04/3{\cdot}59 = 4{\cdot}55$ by Copenhaver & Lardy's procedure.

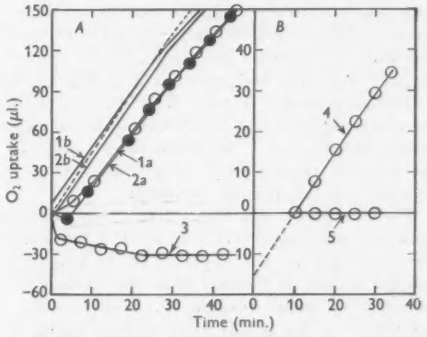

Fig. 3. Measurement of O_2 uptake of the same sarcosomal preparation (sucrose-ethylenediaminetetraacetic acid preparation from rat heart) in the same reaction mixture by Copenhaver & Lardy's procedure (*A*), and by our procedure (*B*). The concentrations of reactants were identical in the two cases, but the reaction volume ordinates differ by a factor of 3. Bath temperature, 30°. *A*. Curves 1*a* and 2*a* show the O_2 uptake calculated directly from the manometric readings of duplicate flasks. Curve 3 is calculated from the pressure changes in a flask treated in the same way but containing only water in the main flask, applying the constant of the manometer used for curve 1. Curve 1*b* is the algebraic sum of curves 1*a* and 3. Similarly, curve 2*b* is the algebraic sum of curve 2*a* and a curve corresponding to curve 3, applying the constant of the manometer used for curve 2. The dotted curve shows the course of the O_2 uptake calculated from *B*. *B*. Curve 4, O_2 uptake from 10 min. to 34 min. by usual procedure. Extrapolation to zero time gives O_2 uptake between 0 and 10 min. Curve 5 is calculated from pressure changes in flask containing only water.

DISCUSSION

Our conclusion that the P:O ratio for the oxidation of α-ketoglutarate to succinate is 3 is at variance with the presently accepted value of 4 (see, for example, Hunter, 1951; Ochoa & Stern, 1952; Krebs *et al.* 1953). It is, however, in agreement with Ochoa's (1944) original assessment.

The difference between our findings and those of other workers could conceivably be due to: (1) a real tissue difference between heart-muscle mitochondria (sarcosomes) and liver mitochondria, which have been used in most of the other work; (2) the method of preparing the mitochondria; (3) the method of measuring the phosphorylation; (4) the method of measuring the oxygen uptake.

(1) The possibility that the P:O ratio for heart-muscle sarcosomes and liver mitochondria are different was given serious consideration, but it now seems unlikely for two reasons: (*a*) Feldott & Plaut (1953), from the same Institute as Copenhaver & Lardy (1952), have obtained with heart preparations P:O ratios which are essentially the same as those found by the latter with liver mitochondria; (*b*) our own limited experience with liver mitochondria, prepared by Copenhaver & Lardy's method, does not suggest any difference between the two preparations (P:O with rat-liver mitochondria, 2·50).

(2) The method of preparation of the sarcosomes has been examined both in previous papers (Cleland & Slater, 1953; Slater & Cleland, 1953*a*, *b*) and in this paper (p. 30). We have varied the procedure considerably without affecting the P:O ratio. The only feature of our procedure differing from those of other workers is the inclusion in the isolation medium of 0·01 M ethylenediaminetetraacetic acid, which either has no effect on the P:O ratio or raises it slightly. Attempts to reproduce as closely as possible the preparative methods of other workers yielded P:O ratios rather lower than obtained by our method.

In our experience, the activity of the α-ketoglutaric oxidase system is a much more sensitive index of the effects of various treatments of the sarcosomes than is the P:O ratio. The activity (Q_{O_2}) of our preparations is considerably higher than those of other heart preparations (see Table 13).

(3) It is clear from the experiments described in Table 7 that our method of measuring the yield of phosphorylation gives the same results as the more commonly used method of measuring the uptake of

Table 13. *Activity of α-ketoglutaric oxidase system in heart-muscle preparations*

Preparation	Animal	Temp. (°)	[Fluoride] (M)	[Malonate] (M)	Q_{O_2}	Reference
Cyclophorase	Rabbit	38	0	0	33–45	Paul *et al.* (1952)
Mitochondria	Guinea pig	30	0	0	72	Plaut & Plaut (1952)
Sarcosomes (sucrose-ethylenediaminetetra-acetic acid)	Rat	25	0	0	163*	This paper
		25	0·04	0·01	79†	
	Cat	25	0·04	0·01	76‡	
	Guinea pig	25	0·04	0·01	52§	

* Calculated from mean value in presence of fluoride and malonate, using data of Table 5.
† Mean of sixteen preparations, standard error of mean = 5·5.
‡ Mean of two preparations (74 and 78).
§ Single preparation.

inorganic phosphate. Further, Table 12 shows that our reaction mixture and that of Copenhaver & Lardy (1952) give the same P:O ratio. Thus, it appears certain that the difference in the methods of measuring the phosphorylation is not responsible for the different P:O ratios obtained by the latter and by ourselves.

(4) The most likely explanation of this difference is the method of measuring the oxygen uptake. The manometric apparatus commonly used for determining the oxygen uptake by tissue preparations was designed primarily for the measurement of rates of oxygen uptake and is not very suitable for the accurate measurement of the actual amount of oxygen consumed when the instability of the system does not permit an adequate period for temperature equilibration before beginning the reaction. This difficulty was recognized by earlier workers on oxidative phosphorylation and various devices were used to allow for the oxygen uptake during the equilibration period. Lehninger & Smith (1949) overcame the difficulty by measuring the amount of oxidized product (acetoacetate) with β-hydroxybutyrate as substrate. This method is reliable only when it is certain that the oxidized product is not further metabolized. Many workers (e.g. Lehninger, 1949; Copenhaver & Lardy, 1952) allow 5 min. equilibration period before commencing the manometric readings. This period is far less than that considered desirable in monographs on manometric techniques; for example, both Dixon (1951) and Umbreit (1949) state that 10–15 min. are necessary. It appears particularly inadequate in the case of Copenhaver & Lardy's (1952) procedure, which involves warming a Warburg flask with two side bulbs from 0 to 30° in 5 min. With apparatus of this type available to us, 5 min. was not sufficient for temperature equilibration and the oxygen uptake was grossly underestimated during the first few minutes. Since the total experimental period used by Copenhaver & Lardy was only 15 min., the error in the calculation of the overall oxygen uptake was serious.

Copenhaver & Lardy (1952) specifically state that 'control experiments demonstrate that the 5 min. pre-incubation period was adequate to obtain thermal equilibrium'. If this is so, the rate of heat transfer in their apparatus was considerably greater than in ours. This may well be the case, and the error in Copenhaver & Lardy's procedure may not be as great as suggested by Fig. 3*A*; for example, the error would have been considerably less in this experiment if a 6 min. instead of a 5 min. equilibration had been given. However, the long time required for the maximum rate of oxygen uptake to be reached and for a steady reading in a control flask containing only water strongly suggests that a considerable error in measuring the oxygen uptake is inherent in Copenhaver & Lardy's procedure. This is supported by the fact that the mean $\Delta O:\Delta$α-Kg in their experiments was 0·9 with fluoride, 1·0 without fluoride compared with 1·13 obtained in our experiments with fluoride. The results shown in Fig. 1 of the present paper clearly show that there is considerable oxidation past succinate.

The strongest argument that the difference between Copenhaver & Lardy's (1952) findings and our own lies in the method of measuring the oxygen uptake is the fact that the mean P:α-Kg obtained by these workers (3·3 with fluoride, 3·2 without fluoride) is only very slightly higher than our values. The P:α-Kg is independent of the manometric measurements.

Unless ethylenediaminetetraacetic acid is used, the combination of the pressure development while the flask is coming to thermal development and the progressive inactivation of the α-ketoglutaric oxidase system could conceivably result in a uniform rate of change of the manometric readings. Thus both the failure of thermal equilibration and the instability of the enzyme system might be overlooked.

The other studies which have obtained a P:O ratio greater than 3 were not as detailed as those of Copenhaver & Lardy (1952). Judah (1951) reported only one value (3·4) in the text, without experimental details. Kielley & Kielley's (1951) method of measuring the oxygen uptake is preferable to that of Copenhaver & Lardy (1952) in that the flask contents were at room temperature before adding the cold enzyme, the reaction temperature was 28° and

the equilibration period was 8 min. The reaction time was also usually somewhat longer than Copenhaver & Lardy's (1952). However, it seems possible that the oxygen uptake is also slightly underestimated in these experiments. The P:O ratio in the presence of malonate and hexokinase was determined with only two preparations, values of 3·1 and 3·5 being obtained. Although Barkulis & Lehninger (1951) state that 'the highest P:O ratios obtained for α-ketoglutarate oxidation were somewhat over 3, confirming Hunter & Hixon', the four values given by these authors range from 2·42 to 3·25, with a mean of 2·89. These values appear to be more in line with a P:O ratio of 3.

Hunter (1951) believes that an observed P:O ratio of 3 indicates a true value of 4·0, since 100% recovery of phosphorylation is not likely even in the presence of fluoride. As already discussed (Slater, 1953*b*; Slater & Holton, 1953), we do not believe that such a conclusion is justified. Provided sufficient hexokinase is added, practically all the ATP will react with this enzyme, and an insignificant proportion with the ATP-ase.

Hunter (1951) also supports the view that the P:O ratio is 4 by his finding (Hunter & Spector, 1951) that dinitrophenol eliminates about 75% of the phosphorylation due to α-ketoglutarate oxidation, leaving 25% intact, which is consistent with the idea that there are three phosphorylations sensitive to dinitrophenol and one that is unaffected. However, this is not strong evidence, since the same result would be obtained if two phosphorylations were completely inhibited and the third inhibited by 25% by the concentration of dinitrophenol used. In fact, Judah (1951) found some inhibition by dinitrophenol of the substrate-linked phosphorylation (measured by the anaerobic dismutation of α-ketoglutarate to glutamate and succinate in the presence of ammonia).

Krebs *et al.* (1953) have also concluded that the P:O ratio in the oxidation of α-ketoglutarate to succinate by rat-liver suspensions is 4. The actual values for the ratio, obtained in the absence of malonate or fluoride, were 3·0–3·2. Since an extrapolation procedure similar to that employed in this paper was used to measure the oxygen consumed in the first few minutes of the experiment it is unlikely that the oxygen uptake was underestimated in the experiments of Krebs *et al.* (1953). The cause of the disagreement probably lies in the method of measuring the rate of esterification of inorganic phosphate. Krebs *et al.* do, in fact, use a method different from other workers in this field, namely the measurement of the rate of incorporation of ^{32}P into ATP. While this method has the advantage of completely eliminating the possibility of errors due to hydrolysis of ATP, there is a serious theoretical objection, which could cause the rate of phosphorylation to be overestimated.

It has been shown by Kaufman (1951) that one of the phosphorylation reactions coupled with the oxidation of α-ketoglutarate to succinate is described by the following reactions (CoA = coenzyme A; DPN^+ = diphosphopyridine nucleotide; DPNH = reduced diphosphopyridine nucleotide):

$$(1)\ \alpha\text{-Kg} + \text{CoA} + DPN^+ \xrightarrow{k_1} \text{succinyl-CoA} + \text{DPNH} + H^+,$$

$$(2)\ \text{succinyl-CoA} + \text{ADP} + H_3PO_4 \underset{k_3}{\overset{k_2}{\rightleftharpoons}} \text{succinate} + \text{CoA} + \text{ATP}.$$

The P:O ratio (one oxygen atom being equivalent to the reduction of one molecule of DPN) of this part of the α-ketoglutaric oxidase system is 1, and in the steady state each molecule of succinyl-CoA formed by reaction (1) would yield 1 molecule of ATP by reaction (2). However, if k_2 and k_3 are both large in comparison with k_1, there would be a rapid exchange between inorganic phosphorus and ATP, at a rate unrelated to the rate of synthesis of ATP. In other words, the rate of synthesis of ATP by reactions (1) and (2) (ignoring subsequent reactions of ATP, for example hydrolysis) is given by the expression rate of synthesis of ATP $= k_2$ [succinyl-CoA] [ADP] [H_3PO_4] $- k_3$ [succinate] [CoA] [ATP], while rate of incorporation of $^{32}P = k_2$ [succinyl-CoA] [ADP] [H_3PO_4]. Thus the rate of incorporation of ^{32}P may greatly exceed the rate of synthesis of ATP, especially when [ATP] is high, as it is in the experiments of Krebs *et al.*

This kinetic treatment is undoubtedly oversimplified, since it is unlikely that termolecular collisions are involved. It is probable that an intermediate, for example CoA-monophosphate, is involved in reaction (2). But the same general conclusion will be arrived at, independently of the detailed mechanism of reaction (2), provided that the overall reaction (2) is reversible, as has been demonstrated by Kaufman (1951).

Reaction (2) is only a particular example of a type of reaction which may be general in oxidative phosphorylation, namely the utilization of the high energy of an intermediate compound to form ATP from ADP and inorganic phosphate. It is likely that the change of free energy in such a reaction is small, i.e. the reaction will be readily reversible. When such reversible reactions involving ATP and inorganic phosphate occur, the rate of incorporation of ^{32}P into ATP will overestimate the rate of oxidative phosphorylation.

Krebs *et al.* find only slow rates of incorporation of ^{32}P into ATP under anaerobic conditions with either α-ketoglutarate or succinate as substrate. How-

ever, experiments of this sort are not adequate controls for the detection of errors of the type just discussed, since the ATP concentration declined rapidly.

A discussion of the mechanism of oxidative phosphorylation is outside the scope of this paper, the purpose of which is to establish the stoicheiometry of the reaction. Our conclusions are described by the equation

$$\alpha\text{-Kg} + \tfrac{1}{2}O_2 + 3ADP + 3H_3PO_4 \rightarrow \text{succinate} + CO_2 + 3ATP + 3H_2O.$$

SUMMARY

1. Variations in the method of preparing heart-muscle sarcosomes had little effect on the yield of oxidative phosphorylation (P:O ratio) coupled with the oxidation of α-ketoglutarate to succinate, although the Q_{O_2} of the α-ketoglutaric oxidase system was considerably affected. The P:O ratio was also little changed if the sarcosomes were mixed with other components of the muscle.

2. For accurate measurement of the P:O ratio, it is necessary to purify the yeast hexokinase preparation sufficiently so that it no longer catalyses an anaerobic reaction of α-ketoglutarate.

3. The further oxidation of succinate is not stopped completely by the addition of 0·01 M malonate and 0·04 M fluoride. Under these conditions, an average of 1·13 atoms of oxygen were consumed per mole of α-ketoglutarate disappearing.

4. The concentration of all components of the reaction mixture can be varied considerably without affecting the P:O ratio.

5. Measurements of the P:O ratio on thirty-two sarcosomal preparations are presented and analysed. In contrast to recent measurements in other laboratories, but in agreement with Ochoa's (1944) original assessment, it is concluded that the P:O ratio for the oxidation of α-ketoglutarate to succinate is 3.

6. It is suggested that the high values obtained by other workers is due in some cases to underestimation of the oxygen uptake, and in one case to overestimation of the rate of phosphorylation.

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The Analysis of Adenosine Triphosphate and Adenosine Diphosphate Preparations by Paper Ionophoresis

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The importance of adenosine triphosphate (ATP) in cell metabolism and the instability of its preparations during storage have resulted in a keen interest being shown in methods for its analysis.

Until recently the most satisfactory analysis of ATP has involved the preparation and use of highly purified enzyme systems (Bailey, 1949). Other less time-consuming methods involving paper chromatography are now available. The most recent (Eggleston & Hems, 1952) involves the separation and estimation of ATP, adenosine 5-phosphate (AMP), orthophosphate and pyrophosphate + adenosine diphosphate (ADP) by 'two-solvent one-dimensional chromatography' and the individual determination of pyrophosphate by a further chromatographic separation.

During the development of a general scheme for the analysis of acid-soluble phosphates a method involving paper ionophoresis was evolved which was found to separate all the above esters in one operation and in the space of a few hours. The technique which is described below has been applied to the analysis of seven samples of ATP and one of ADP.

EXPERIMENTAL

Ionophoreses were carried out on 28 cm. widths of Whatman no. 3 paper washed three times with N formic acid and finally with distilled water that had been passed through Amberlite Monobed Resin MB–1 analytical grade (supplied by British Drug Houses Ltd.). Twelve sheets (28 × 45·25 cm.) were supported by a perforated sheet of Perspex built into a Perspex box so that suction could be applied from beneath the paper drawing the solvents through it. The sheets were dried in a current of warm air. This treatment reduced the phosphorus of the paper to about 0·03 μg. P/sq.cm., and removed interfering substances.

The electrodes were two lengths of 26 (S.W.G.) gauge copper wire threaded through holes pierced at 1 cm. intervals 1·3 cm. from each edge (Fig. 1). The wires extended beyond the paper and provided a means of suspending it during subsequent operations.

As the method suffers no interference from Ca^{2+} or Ba^{2+} the samples were applied as aqueous solutions acidified, in the instance of the Ba salts, with N-HCl. (If the sample is freshly precipitated as the Ba salt and in a finely divided state it can be applied as the suspension.) The solution (2·5 μl. containing about 25 μg. P) was applied to the paper 1·9 cm. from the cathode. This large quantity was necessary in order to provide measurable amounts of the weaker components.

5% (w/v) Oxoid agar (supplied by Oxo Ltd.) and the same solution containing 5% (w/v) NaCl were delivered, in a molten state, on to the cathode and anode, respectively, to ensure intimate association of wire and paper and to provide at the anode a reservoir of Na ions.

The paper was sprayed with aqueous solution (pH 3·2) containing 4·6% (v/v) redistilled *n*-butyric acid and 0·057% (w/v) NaOH until it was just saturated with the solution. The paper was then suspended from the edge of a covered glass trough over sufficient water to maintain a saturated atmosphere.

A potential of 300–400 v (d.c.) was applied to give a current of 0·25 mA/cm. breadth. After 2 hr. the paper was withdrawn and dried in a current of warm air and the phosphates were located by spraying first with 0·1% (w/v) $FeCl_3$, $6H_2O$ in 80% (v/v) aqueous ethanol and secondly, after drying, with 1% (w/v) sulphosalicylic acid in 80% (v/v) ethanol (Wade & Morgan, 1953).

The spots were cut from the paper, transferred to $\frac{4}{5}$ in. × $\frac{1}{2}$ in. Pyrex test tubes and ashed on a microdigestion rack (Gallenkamp & Co. Ltd., London). After cooling, 0·5 ml. 72% (w/v) aqueous $HClO_4$ was added and digestion carried out using initially the minimum heat necessary from the bottom element to boil the acid and finally heat from both elements to enable the condensing acid to reach the upper parts of the tubes.

When digestion was complete the tubes were cooled. 1 ml. 5N-NaOH and 1 drop of 0·05% (w/v) phenolphthalein in 80% (v/v) aqueous ethanol were added to each and 20% (w/v) aqueous $HClO_4$ was added until the indicator was decolorized. Orthophosphate determinations were carried out by the method of King (1932), and the quantities of reagents were adjusted to give a final volume of 5 ml. The colour intensities were read on a Spekker absorptiometer (A. Hilger & Sons Ltd., London) using microcells of 2 cm. depth and 1 ml. capacity.

In the analysis of an ATP or an ADP preparation 5 mg. were treated with 0·1 ml. water and acidified where necessary with the minimum quantity of N-HCl. 2·5 μl. quantities were delivered alternately on to the base line (1·9 cm. from the cathode) of an ionophoresis paper (28 cm. long and 15 cm. broad) at 2·5 cm. intervals, and on to squares (0·63 cm. × 0·63 cm.) of filter paper. Three spots were applied to the ionophoresis paper and four to the paper squares. The latter were subsequently digested to determine the total P applied; blank squares similarly treated provided reagent blanks.

After ionophoresis and the detection of the phosphates, 2·5 cm. strips of the paper containing the components of each original spot were cut out and divided into rectangles

containing the individual spots. Each rectangle was digested and total P determined; from this was subtracted the quantity of P calculated from the blank to be present in the area of paper digested.

Special precautions. As other workers have stressed, it is imperative to have paper and reagents as free as possible from interfering substances. Glass-distilled water is recommended for use throughout. Unsatisfactory conditions are reflected in the nature of the ATP spot, which streaks badly.

The even spraying of the solvent is important and requires some practice. The application of more solvent than the paper can accommodate should be avoided as this results in diffused spots and a merging of the ATP spot with the orthophosphate spot. If this continues to give trouble the following alternative form of analysis can be adopted. The spot of ATP and orthophosphate are combined and extracted with 0·1 N-HCl. Orthophosphate and total P in this extract are then determined. This alternative is made possible by the mild conditions employed in the location of the phosphates.

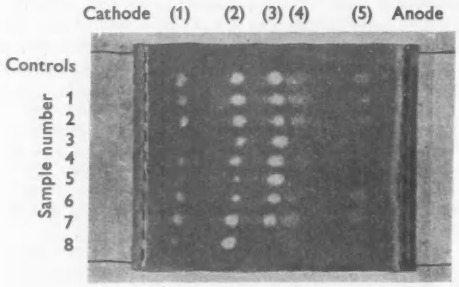

Fig. 1 (i)

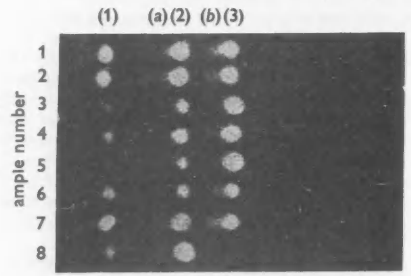

Fig. 1 (ii)

Fig. 1. Fractionation of ATP and ADP preparations by paper ionophoresis. Sample numbers correspond to those in Table 1. The total P applied to the paper was adjusted to give about 10 μg. P in the main constituent. (1) AMP; (2) ADP, (3) ATP, (4) orthophosphate, (5) pyrophosphate. (i) Photograph of an ionophoresis paper after detection of the phosphates by $FeCl_3$/sulphosalicylic acid spraying. (ii) Photograph of an ultraviolet contact print of an electrophoresis paper showing the presence of two ultraviolet absorbing areas (*a*) and (*b*) in addition to those of (1), (2) and (3).

Table 1. *Analyses* of preparations of adenosine triphosphate and diphosphate* (ATP *and* ADP)

Preparation	Source	Storage temp.	Year received	Estimated as % total P					
				AMP	ADP	ATP	Ortho-phosphate	Pyro-phosphate	Total estimated
1. ATP, free acid	Light & Co. Ltd.	Room	1950	10·7±0·24†	27·3±0·30	28·9±0·26	24·5±0·12	7·8±0·30	99·2±0·56
2. ATP, Ca salt	Light & Co. Ltd.	Room	1950	10·9±0·78	27·8±1·03	31·5±1·01	24·3±0·90	7·9±0·74	102·4±2·02
3. ATP, Ba salt	Private	2°	1952	4·1±0·39	18·3±0·59	64·4±1·45	8·8±0·18	2·3±0·72	97·9±1·78
4. ATP, Ba salt	Light & Co. Ltd.	2°	1952	7·3±1·25	26·3±0·80	50·8±1·14	10·6±0·51	2·1±0·51	97·1±2·01
5. ATP, Ba salt	Schwarz Lab. Inc.	2°	1952	3·4±0·94	7·5±0·83	74·0±2·04	4·1±0·53	4·2±0·57	93·2±2·52
6. ATP, Na salt	Boots Pure Drug Co.	Room	1945	8·8±0·33	13·7±0·26	37·3±1·43	15·3±0·41	23·6±0·37	98·7±1·59
7. ATP, Ca salt	Light & Co. Ltd.	Room	1950	11·2±0·23	24·9±0·40	28·2±0·43	23·3±0·39	6·5±0·24	94·1±0·77
8. ADP, Ba salt	Schwarz Lab. Inc.	2°	1952	4·9±0·29	76·0±1·18	3·8±0·14	10·8±0·21	—	95·5±1·24

* Analyses were carried out in March 1953.
† Standard deviation.

RESULTS

The analyses given in Table 1 represent the average of three sets of determinations carried out at the same time. Fig. 1 (i) shows the spots of AMP, ADP, ATP, orthophosphate and pyrophosphate separated by the ionophoresis of seven preparations of ATP and one of ADP.

Low concentrations of other phosphoric esters are present in most of the preparations analysed. At least two further ultraviolet-absorbing esters are present in preparations 1, 2, 4, 6 and 7 (Fig. 1 (ii)). One (*a*) occurs between AMP and ADP; its identity is unknown and it has not been included in this analysis. The other (*b*) is located just behind the ATP spot, and contaminates it.

DISCUSSION

The elementary analysis of ATP and ADP preparations, together with the estimation of acid-labile phosphate, is still used by some workers as a criterion of purity (LePage, 1945; Dounce *et al.* 1948). Other workers seeking more specific methods have used enzyme systems (Kalckar, 1943, 1944, 1947; Bailey, 1949; Rowles & Stocken, 1950); such methods, however, require the lengthy preparation of highly purified enzymes. In addition to this, discrepancies are reported to exist in the results obtained from the use of different combinations of enzymes (Rowles & Stocken, 1950) and the reactions catalysed by some of them are still questioned (Bailey, 1949; Banga, 1947). This type of analysis, in common with some others, requires the initial preparation of the free acid or the sodium salt of the ester, and if losses in ATP of up to 20 % incurred by the direct precipitation of the barium as the sulphate are to be avoided, an ion-exchange or electrical transport system has to be used to bring this about (Bailey, 1949).

Ion-exchange resins have been used by Cohn & Carter (1950) for the separation and analysis of adenine, adenosine, AMP, ADP and ATP. As an analytical method this procedure suffers the disadvantage of requiring the use of large volumes of fluid. These workers also carried out this separation by paper chromatography using an *iso*amyl alcohol : disodium phosphate : water system. In both methods barium was removed as the sulphate and no account was taken of any pyrophosphate that may have been present.

The Tiselius apparatus has also been used for the ionophoretic separation of these adenine derivatives (Bock & Alberty, 1951); in this method no provision was made for the analysis of pyrophosphate or orthophosphate.

A paper-chromatographic technique, recently introduced by Eggleston & Hems (1952), involves 'two-solvent, one-dimensional chromatography' and overcomes some of the disadvantages of the above methods. The apparatus required is simple, only small quantities of material being necessary, and the analysis is speedy. However, conversion of the preparations to the sodium salts is still necessary, and an individual determination has to be made for the pyrophosphate.

The main advantages of the method described here can be outlined as follows. The apparatus is very simple and inexpensive to construct; the analysis can be carried out completely inside a working day; no interference is caused by the presence of barium or calcium ions, thus permitting the direct analysis of any of the common forms of preparations; and the separation of mixtures into AMP, ADP, ATP, orthophosphate and pyrophosphate can be carried out in one operation.

The method, as described, cannot claim to analyse these phosphates in preparations that are heavily contaminated with other esters but it is put forward as a basic technique to which can be added more exacting steps involving the elution and subsequent analysis of the fractions separated.

SUMMARY

1. A method is described for the separation of adenosine triphosphate, adenosine diphosphate, adenosine 5-phosphate, orthophosphate and pyrophosphate by paper ionophoresis.

2. The ionophoresis technique differs from those normally used in having no reservoirs of buffer at the electrodes.

3. The method has been used in the analysis of seven samples of adenosine triphosphate and one of adenosine diphosphate. The results of this are given.

We are indebted to Dr D. Herbert for helpful criticism of this paper and to Dr K. Bailey for supplying a sample of adenosine triphosphate. The statistical treatment of results was carried out under the guidance of Mr S. Peto. Acknowledgement is made to the Ministry of Supply for permission to publish this paper.

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The Distribution of Ascorbic Acid between the Particulate and Non-particulate Components of Adrenal and Liver Cells

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For a number of years cytologists have been interested in the distribution of ascorbic acid within the cell. For the demonstration and localization of the acid a technique has been devised which depends on its ability to reduce silver nitrate to metallic silver, and convincing evidence has been presented for considering this technique specific for ascorbic acid (see Bourne, 1936). Deposition of metallic silver in the region of the Golgi apparatus and on mitochondria has been taken to indicate that the ascorbic acid may be present in these structures (Bourne, 1936, 1951).

Barnett & Fisher (1943) applied the silver nitrate technique to pieces of a gelatin medium in which ascorbic acid and olive oil had been thoroughly mixed prior to its setting. They observed aggregation of metallic silver particles at the interfaces between gelatin on the one hand and oil droplets or dust particles on the other; similar aggregation was seen on the surface of fragments of powdered glass that had been suspended in a gelatin medium containing ascorbic acid. Barnett & Fisher (1943) concluded from this that the concentration of metallic silver at the surfaces of mitochondria does not necessarily indicate that the ascorbic acid is concentrated in or on these bodies. Palade & Claude (1949*a*, *b*) have since brought forward weighty evidence in favour of the view that the Golgi apparatus is an artifact produced by the coalescence of intracellular phospholipids with the formation of characteristic patterns. If this view is accepted, the question of the association of ascorbic acid with Golgi material is no longer significant.

It was thought desirable to study the distribution of ascorbic acid in cytoplasmic fractions obtained by high-speed centrifugation of tissue dispersed in sucrose solutions at low temperatures. Most experiments were carried out with preparations obtained from ox adrenal medulla but a few observations on ox adrenal cortex and on dog liver are also reported.

METHODS

Preparation of tissue fractions. Ox adrenal glands from the abattoir were brought to the laboratory as soon as possible after the animal had been killed; the medulla and cortex were immediately dissected apart. Liver tissue was obtained fresh from dogs which had been bled under ether anaesthesia. The tissues were dispersed in ice-cold 0·25 M sucrose solution with a Potter-Elvehjem homogenizer. Sufficient sucrose solution was added to give a final concentration of 10 g. of tissue in a total volume of 100 ml. A Measuring and Scientific Equipment Ltd. (M.S.E.) refrigerated centrifuge set at $-1°$ was used in these experiments. The initial dispersion was centrifuged for 30 min. at 950 *g* (at the bottom of the tube) in order to throw down incompletely broken cells, cell nuclei, blood cells and any tissue which might have escaped disintegration. The supernatant fluid from this preliminary centrifugation served as the starting material for further fractionation. A portion was centrifuged at 22000 *g* for 30 min.; this produced an almost clear supernatant fluid, indicating complete sedimentation of particulate matter. This supernatant was poured off and an equal volume of 0·25 M sucrose solution was added, in which the sedimented particles were resuspended. Throughout these manipulations all vessels were kept in a water bath containing ice and every effort was made to maintain the temperature of the solutions below 4°. In one experiment with dog liver the tissue was dispersed in 0·88 M sucrose solution. The supernatant from the low-speed centrifugation and both supernatant and resuspended sediment from the high-speed centrifugation were assayed for their ascorbic acid content.

Estimation of ascorbic acid. A modification of the method described by Bessey (1938) was used. To 2 ml. of each fraction 23 ml. of 3% metaphosphoric acid were added; this solution was filtered and the pH of the filtrate was adjusted to 3·7 with citrate buffer. Equal quantities of the buffered extract and 1·6% (w/v) 2:6-dichlorophenol indophenol solution were mixed and the percentage transmission read at 518 mμ. in a Unicam spectrophotometer; the increase in transmission was proportional to the ascorbic acid concentration. In one experiment ascorbic acid was re-estimated after bubbling H_2S through the buffered extract for 15 min., followed by nitrogen for 1 hr., in order to obtain a measure of any dehydroascorbic acid present.

RESULTS

The ascorbic acid content of each fraction is shown in Table 1. The figures for the ascorbic acid content of the low-speed supernatants indicate that the bulk of the content of the tissues is present in this fraction. Data on the ascorbic acid content of the ox adrenal gland are given by Harris & Ray (1933). They found 1·1 to 1·2 mg./g. of tissue. The figures given in Table 1 for the suspension range from 0·47 to 1·17 mg. The figure of 1·17 mg. of ascorbic acid/g. of adrenal cortex is about two-thirds of the value

Table 1. *Distribution of ascorbic acid between the particulate and non-particulate fractions of adrenal and liver cells*

(For details see text)

Tissue	Ascorbic acid content: Supernatant from low-speed centrifugation (Calc. as mg./g. fresh tissue) (*a*)	Supernatant from high-speed centrifugation (Calc. as mg./g. fresh tissue) (*b*)	Supernatant from high-speed centrifugation (Calc. as % of value in *a*) (*c*)	Sediment from high-speed centrifugation (Calc. as mg./g. fresh tissue) (*d*)	Sediment from high-speed centrifugation (Calc. as % of value in *a*) (*e*)
Ox adrenal medulla	0·85	0·80	94	0	0
Ox adrenal medulla	0·47	0·41	87	0·06	14
Ox adrenal medulla	1·17	1·19	102	0	0
Ox adrenal medulla	0·77	0·64	84	0·07	10
Ox adrenal cortex	1·17	1·12	96	0	0
Dog liver					
Dispersed in 0·25 M sucrose	0·27	0·24	90	0·04	14
Dispersed in 0·88 M sucrose	0·43	0·39	91	0·05	11
Ox adrenal medulla					
Before treatment with H_2S	0·85	0·80	—	0	—
After treatment with H_2S	0·79	0·80	—	0·02	—

(1·85 mg.) given by Birch, Harris & Ray (1933). The finding that treatment with hydrogen sulphide did not increase the figures for the ascorbic acid content is in agreement with the findings of Bourne (1936) and Sayers & Sayers (1948) that in the adrenal gland all the ascorbic acid is in the reduced state.

In all the tissues examined the greater part of the ascorbic acid, 87 % to 100 %, was found in the supernatant from the high-speed centrifugation; the redispersed particles contained only 0 % to 14 % of the total amount of ascorbic acid.

In the procedure used in these experiments the use of 0·25 M sucrose resulted in the sedimentation of all the particulate components of the cytoplasm; on the other hand, it is known that in 0·88 M sucrose the smallest particles, the microsomes, remain in suspension. The 0·88 M sucrose was used in one experiment with dog liver; the conclusion that in this experiment the microsomes were not sedimented is supported by the observation that the supernatant fluid was opaque. There was no difference in the distribution of ascorbic acid in fractions obtained using either medium.

Some of the fractions obtained were treated with silver nitrate solution as described by Bourne (1936).

These fractions were: (1) the supernatant from the low-speed centrifugation, (2) the resuspended sediment from the high-speed centrifugation and (3) the same resuspended sediment with ascorbic acid (neutralized with NaOH) added in a final concentration of 0·1 mg./ml. To 2·0 ml. of each of the samples was added 0·1 ml. of 10 % (w/v) $AgNO_3$ in 10 % (v/v) acetic acid. A drop was placed on a microscope slide covered with a glass cover square and examined within 10–15 min.

Microscopic examination showed no staining of the resuspended mitochondria (2), but they stained brown when suspended in a medium containing sodium ascorbate (3). Particles were similarly stained in the sample (1) derived from low-speed centrifugation.

DISCUSSION

The findings show that in the dispersions used in these experiments little if any ascorbic acid was found in the cytoplasmic particles. The small amount sometimes found in the granular fraction can probably be attributed to contamination with small quantities of supernatant fluid. These findings raise the question whether in the living cell the ascorbic acid is present in the mitochondria or in the cytoplasmic sap. This question cannot be definitely answered on the basis of the experiments given here since the possibility remains that ascorbic acid associated with mitochondria could become detached in the preparation of the various fractions. However, other low-molecular weight components of the adrenal medulla are retained by the particles during this procedure (Welch & Blaschko, 1953).

Evidence that ascorbic acid is present in the granules of cells of adrenal medulla rests on the demonstration of precipitates of metallic silver in association with mitochondria when an acid silver nitrate solution is applied to medullary tissue (Bourne, 1936). However, a precipitate can occur on the surface of granules free from ascorbic acid when they are suspended in a medium containing ascorbic acid. This shows that the localization of the silver precipitate gives no indication of the distribution of ascorbic acid in the living cell.

SUMMARY

1. Estimations have been made of the ascorbic acid content of the particulate and non-particulate fractions of cytoplasm separated by high-speed centrifugation of sucrose dispersions of ox adrenal medulla and adrenal cortex, and of dog liver.

2. The ascorbic acid was found to be entirely or almost entirely in the non-particulate fraction.

3. These results are discussed and additional evidence is advanced for considering that the location of reduced silver particles does not necessarily indicate the localization of ascorbic acid within the cell.

I wish to express my thanks to Dr H. Blaschko for his advice and criticism throughout the course of this work, and to Prof. J. H. Burn for allowing me to work in his Department during the tenure of a C. J. Martin Fellowship of the Australian National Health and Medical Research Council.

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Biosynthesis of Fatty Acids by Slices and Cell-free Suspensions of Mammary Gland

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The experiments to be described were undertaken with the purpose of developing, if possible, cell-free enzyme preparations suitable for the study of the biosynthesis of fatty acids *in vitro*. The lack of even crude preparations of this kind until recently is probably responsible in a large measure for the paucity of detailed knowledge concerning the intermediary stages of fat metabolism.

While our work was in progress Gurin and his colleagues reported that 'homogenates' and some soluble enzyme preparations made from pigeon liver (Brady & Gurin, 1952*a*, *b*) and rat liver (Dituri & Gurin, 1953) synthesize long-chain fatty acids from [^{14}C]acetate. In our experiments, we have used the mammary gland of lactating animals, which is known to be very active in synthesizing fat (cf. reviews by Folley, 1952; Popják, 1952*a*, *b*). The mammary gland of lactating rats was used mostly, but some experiments were also carried out with sheep udder tissue. The results described here have already been communicated in a preliminary form (Popják & Tietz, 1953*a*).

MATERIAL AND METHODS

Lactating rats, 7–14 days after parturition, were anaesthetized with ether and killed by dislocation of the neck. The abdominal pair of the mammary glands was removed together with the overlying skin and packed in crushed ice. When the glands were thoroughly chilled, they were dissected and freed from connective tissue. One rat provided usually 5–6 g. of gland.

The sheep udder tissue, one portion packed in ice and another in solid CO_2, was transported from the slaughter house to the laboratory. The experiments with the cell-free suspensions prepared from the ice-cold tissue were set up 4 hr., and those with the slices 5 hr., after the killing of the sheep. The experiments with rat tissue were usually started 2–3 hr. after the death of the animals. (We are indebted to Dr S. J. Folley, F.R.S., for supplying the udder tissue from a lactating sheep.)

Preparation of tissue slices. Tissue slices were cut with a Stadie-Riggs (Stadie & Riggs, 1944) microtome and were kept in oxygenated ice-cold buffer until used. About 250 mg. of slices were incubated in 3 ml. of phosphate-saline (Krebs & Eggleston, 1940).

Preparation of suspensions. The tissues were minced finely with scissors and the mince was disintegrated for about 2 min. with 2 vol. of ice-cold buffer (0·154 M-KCl, 100 parts; 0·154 M-$MgCl_2$, 10 parts; 0·1 M-potassium phosphate buffer, pH 7·4, 35 parts) in a glass homogenizer tube provided with a loosely fitting pestle (difference between the diameter of the tube and that of the pestle, 0·40 mm.) which was driven by an electric motor. The suspension thus produced was filtered through four layers of gauze into another homogenizer tube with a tightly fitting pestle (difference between the diameter of the barrel and pestle, 0·10 mm.) and was ground further by hand. Large cell fragments and cell nuclei were removed by centrifugation at 400 *g* at 0° for 10 min. The fat which separated on the surface was removed and the supernatant fluid filtered through a small pad of cotton wool. This filtered supernatant, which contained 50 ± 5 mg. dry

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matter/ml. (corrected for salts but not for fat), was used in the experiments. Neither intact cells nor cell nuclei in the suspensions could be identified with the phase-contrast microscope; with the exception of some fat globules, most of the particles showed an intense Brownian movement when viewed with an oil-immersion lens. When suspensions from the sheep udder frozen in solid CO_2 were required, the frozen tissue was first broken up into a fine powder in a stainless-steel mortar chilled with solid CO_2. The powder was then added to the buffer solution in the glass homogenizer tube; the rest of the procedure was as for the fresh tissues.

Incubations. All incubations (slices and suspensions) were made at 37·5° for 3 hr. in Warburg vessels, the centre well of which contained 0·2 ml. of 20% (w/v) KOH. The gas phase was O_2 or air. In the experiments with suspensions, the 'additions' were first measured into the flasks and the volume made up to 1 ml. with water; 2 ml. of suspension were then added and the incubations started immediately. In every experiment, except when the endogenous O_2 consumption of the preparations was being measured, acetate (containing 5 μc of [*carboxy*-^{14}C]acetate, obtained from the Radiochemical Centre, Amersham, Bucks) was added to the incubation mixture in a final concentration of 0·02 M, equivalent to 60 μmoles of acetate/flask. Other substrates (denoted by 'Additions' in the Tables) were added in the following final concentrations: glucose, 0·3%; adenosine triphosphate (ATP), 0·01 M; pyruvate, oxaloacetate, citrate, α-oxoglutarate, succinate, malate and glutamate, 0·02 M.

Oxygen consumption. The values of $-Q_{O_2}$ (μl. O_2/mg. dry wt./hr.) reported are based on the total O_2 consumed during the first hour of incubation and do not represent just the initial rates of O_2 consumption.

Collection of CO_2 for measurement of oxidation of [^{14}C]-acetate. The filter paper and alkali from the centre well of the flasks were added to 2 ml. of freshly boiled water in a centrifuge tube and the centre well was washed out five times with CO_2-free water. One ml. of a 0·5% (w/v) solution of Na_2CO_3 was added to the centrifuge tube as 'carrier' and the contents of the tube were covered with a layer of high-boiling-point petroleum to prevent absorption of atmospheric CO_2. After allowing several hours for the equilibration of the carbonate in the filter paper with the solution (it is extremely difficult to wash out all the alkali from the paper), the paper was withdrawn and washed with CO_2-free water. The carbonate was precipitated as $BaCO_3$ by the addition of several drops of a saturated solution of $BaCl_2$. The $BaCO_3$ was centrifuged and washed three times with freshly boiled water and twice with acetone and was plated for radioactive counting by filtration on to sample disks as described earlier (Popják, 1950).

Extraction of fatty acids and removal of [^{14}C]acetate from the fatty acids. The contents of the incubation flasks were saponified overnight with 1 ml. of 40% KOH on a boiling-water bath. In the experiments with slices the unsaponifiable matter was always extracted, but with the suspensions, in which the unsaponifiable matter amounted to only about 1% of the total fatty acids and which were found to contain only negligible amounts of radioactivity, this extraction was omitted. The hydrolysis mixture was acidified and extracted with 2 vol. of light petroleum (b.p. 40–60°).

In our earliest experiments the fatty acids were purified from contaminating [^{14}C]acetate by washing the light petroleum extract with 0·1 N acetic acid and then with water. Although this technique was effective in removing all but traces of [^{14}C]acetate, it was extremely tedious and unsatisfactory because an appreciable amount of the short-chain fatty acids present in the mammary gland preparations was inevitably lost. A chromatographic technique was therefore resorted to. Dr A. J. P. Martin and Miss Joan Webb of this Institute have devised for the separation of *n*-butyric, propionic, acetic and formic acids a partition-chromatographic column (cf. Cornforth, Hunter & Popják, 1953), in which 0·5 N-H_2SO_4 supported by Hyflo Supercel forms the stationary phase and $CHCl_3$ (saturated with 0·5 N-H_2SO_4) the developing solvent. All fatty acids higher than propionic emerge from this column as a single narrow band. In practice, for the quantities of fatty acids obtained in a single experiment (25–50 mg.) a glass tube of 2 cm. diameter and fitted with a sintered-glass disk was packed with a $CHCl_3$ suspension of Hyflo Super-cel (25 g.) wetted with 0·5 N-H_2SO_4 (20 ml.). The light petroleum solution of the fatty acids (see above) was concentrated to about 1 ml., taken up in 5 ml. of $CHCl_3$ and transferred to the column. After the fatty acids were washed into the column with two further 5 ml. portions of $CHCl_3$, 80 ml. of solvent were allowed to percolate through. The eluted fatty acids were titrated under N_2 with 0·01 N-ethanolic NaOH with bromthymol blue as indicator. The results were very well reproducible; in replicate analyses on the same cell-free suspension the titration values did not vary by more than 5%. When 5 μc of [^{14}C]acetate were added to 50 mg. of higher fatty acids and the mixture chromatographed the latter gave 1–3 counts/min. above background. The acetic acid could be eluted from the columns after passing through about 1 l. of 10% (v/v) *n*-butanol in $CHCl_3$. Although these columns could be used repeatedly after removal of the [^{14}C]acetic acid, it was found more economical on account of the large volumes of solvent required to collect only the fatty acids higher than acetic and to discard the columns after using once.

Resolution to total fatty acids into individual acids by chromatography. The fatty acids were first fractionated by the reversed-phase partition chromatography technique of Howard & Martin (1950), the first solvent applied to the column being 45% (v/v) aqueous acetone. The first band emerging with this solvent contains all short-chain fatty acids up to and including octanoic; decanoic acid appears as the second band. On changing the solvent to 55% acetone, dodecanoic and tetradecanoic acids are eluted successively; 65% acetone is used to remove hexadecanoic (palmitic) acid and 75% acetone to remove octadecanoic (stearic) acid. Any monoethenoid acid will appear in this system with the saturated fatty acid having two carbon atoms fewer. Thus oleic (octadec-9-enoic) is eluted with palmitic acid and palmitoleic (hexadec-9-enoic) with myristic (tetradecanoic) acid. Of the unsaturated acids only oleic acid is present in significant amounts in our preparations and we separated it from palmitic acid by subjecting the mixture of the two (the band emerging with 65% acetone) to hydrogenation in ethyl acetate with 5% palladium on charcoal as catalyst at 20° for 24 hr. The fatty acids were then re-chromatographed and the stearic acid thus obtained represented the oleic acid in the original mixture. The hydrogenation was repeated on the palmitic acid fraction thus freed from oleic acid, but no stearic acid was formed during the second hydrogenation. The reversed-phase chromatographic columns were loaded with not more than 2 mg. total fatty acids/g. packing.

The short-chain fatty acids (45% acetone fraction) were further resolved on a column buffered with 2M-K_2HPO_4:2M-KH_2PO_4 (6:1), pH 8·0. Caprylic, caproic, valeric and butyric acids can be separated on this column which is essentially the same as that already described (Cornforth *et al.* 1953) for the separation of valeric and butyric acids, except that the pH of the present column is higher.

Assay of ^{14}C. Whenever sufficient material was available, the radioactivity of the fatty acids was measured on the sodium salts in infinite thickness samples of 2 cm.^{2} area. However, in the experiments with suspensions, this was seldom possible and the counts had to be taken on thin samples. For this purpose the sodium soaps, dissolved in 0·05–0·10 ml. water, were pipetted on to plastic disks lined with lens paper. An experimental self-absorption curve was prepared for [^{14}C]soaps plated with lens paper. The counts obtained on the thin samples were then corrected to infinite thickness with the help of this curve. The specific radioactivities of the carbon of the fatty acids (Table 10) were calculated as described earlier (Cornforth *et al.* 1953).

From the specific radioactivity and the amount of fatty acids found, the total amount of acetate converted into fatty acid was calculated.

RESULTS

The effect of substrates on fatty-acid synthesis and oxidation of [carboxy-^{14}C]*acetate by mammary gland slices*

As a preliminary to the work with cell-free suspensions, the response of tissue slices to various substrates was tested in the hope that the information thus gained might prove useful in the later experiments. It has been shown by Folley & French (1950) and by Balmain, Folley & Glascock (1952) that rat mammary-gland slices do not utilize acetate for fatty-acid synthesis when acetate is the only substrate in the incubation medium; however, when glucose is also added, acetate is readily used. Slices of sheep udder on the other hand can synthesize fatty acids from acetate without any addition, although glucose causes a further stimulation in this tissue also.

These conclusions of Folley and his collaborators have been fully confirmed (Table 1) and it was found

Table 1. *Fatty-acid synthesis by rat and sheep mammary-gland slices from* [carboxy-^{14}C]*acetate*

(Each flask contained 60 μmoles of acetate (5 μc ^{14}C); 250 mg. of slices and 2·5 ml. of phosphate-saline, pH 7·4. Additions were as indicated (final concentrations in brackets). Final vol. 3 ml. Results are expressed as μmoles of acetate incorporated into fatty acids/100 mg. of initial dry weight of slices.)

		Fatty-acid synthesis					
Expt. no.	Species	None	Glucose (0·3%)	Pyruvate (0·02M)	Oxalo-acetate (0·02M)	α-Oxo-glutarate (0·02M)	Succinate (0·02M)
66	Rat	0·014	7·45	—	—	—	0·113
67	Rat	0·018	10·65	—	0·331	0·14	0·166
76	Rat	0·016	—	0·032	0·087	0·13	—
77	Rat	0·006	9·34	0·258	0·252	0·068	—
96	Sheep	0·293	3·67	1·26	1·18	1·62	2·54

Table 2. *Oxygen consumption and oxidation of acetate by rat and sheep mammary-gland slices in the presence of various substrates*

(Concentrations of substrates and other conditions as in Table 1.)

Expt. no.	Species	Additions	$-Q_{O_2}$	Acetate oxidized (μmoles/100 mg. initial dry wt.)
66	Rat	None	1·49	0·107
		Glucose	4·30	0·654
		Succinate	4·33	0·427
76	Rat	None	0·85	0·327
		Pyruvate	3·97	0·546
		Oxaloacetate	3·23	0·202
		α-Oxoglutarate	2·85	1·770
77	Rat	None	0·74	0·186
		Glucose	4·24	1·280
		Pyruvate	4·15	1·440
		Oxaloacetate	4·83	0·556
		α-Oxoglutarate	2·68	1·960
96	Sheep	None	1·46	4·730
		Glucose	1·56	4·680
		Pyruvate	2·10	4·550
		Oxaloacetate	3·02	1·730
		α-Oxoglutarate	2·14	1·570
		Succinate	5·92	5·910

in addition that oxidizable substrates other than glucose also stimulated fatty-acid synthesis from acetate. As can be seen from the results shown in Table 1 rat mammary-gland slices incorporated only traces of ^{14}C into fatty acids when [*carboxy*-^{14}C]-acetate was the only substrate added to the medium. The addition of pyruvate, oxaloacetate, α-oxoglutarate or succinate, however, caused a significant utilization of acetate for fatty-acid synthesis although not as great as was found in the presence of glucose. In slices of sheep udder, pyruvate and the members of the citric acid cycle were nearly as effective stimulants as glucose and were far more active than in rat tissue.

The difference in behaviour of the mammary-gland slices of the two species was apparent also in their ability to oxidize acetate. As can be seen from the results shown in Table 2 rat mammary-gland slices oxidized only about one-fortieth of the amount oxidized by sheep-udder slices when acetate was the only substrate added. Even in Expt. 77 (Table 2) when the highest oxidation of acetate by rat mammary-gland slices was observed in the presence of glucose, pyruvate or α-oxoglutarate, the levels of oxidation were lower than that found with sheep tissue when incubated with acetate alone. α-Oxoglutarate in the rat tissue stimulated and in the sheep-udder slices inhibited oxidation of acetate. Oxaloacetate, which stimulated the oxygen consumption and fatty-acid synthesis in the slices from both species, had very little effect on acetate oxidation in the rat slices and was inhibitory in the ruminant tissue. Similar effects of oxaloacetate (stimulation of fatty-acid synthesis from acetate and inhibition of acetate oxidation) have been observed in ovarian tissues from the laying hen (Popják & Tietz, 1953*b*).

Fatty-acid synthesis from [carboxy-^{14}C]*acetate by cell-free suspensions of rat mammary gland*

Suspensions, like slices, of rat mammary gland incorporated only traces of acetate into fatty acids when acetate, or acetate and ATP, were the only substrates added to the incubation medium. Glucose either alone or in combination with ATP did not promote fatty-acid synthesis in suspensions, in contrast to its behaviour in slices (Table 3).

The highest rate of incorporation of acetate into fatty acids by these suspensions has so far been observed in the presence of oxaloacetate and ATP (Table 4). Oxaloacetate alone showed a slight stimulation in most suspensions, but really significant fatty-acid synthesis occurred only when ATP was also added. The amount of ^{14}C incorporated into fatty acids by the most active suspensions under these conditions was about 50% of that used by slices in the presence of oxaloacetate. Heating for 5 min. in a boiling-water bath abolished completely the ability of the suspension to incorporate [^{14}C]-acetate into fatty acids.

Table 3. *Fatty-acid synthesis from* [carboxy-^{14}C]-*acetate by suspensions of rat mammary gland in presence of glucose and ATP*

(The results are expressed as μmoles × 10^{-3} of acetate incorporated into fatty acids/100 mg. dry weight of suspension.)

Additions			
None	ATP (0·01 M)	Glucose (0·3%)	Glucose + ATP
3·79	2·72	4·76	5·43
3·09	3·09	3·20	1·05

Table 4. *Effect of oxaloacetate and ATP on fatty-acid synthesis from* [carboxy-^{14}C]*acetate by suspensions of rat mammary gland*

(The results are expressed as μmoles × 10^{-3} of acetate used for fatty acid synthesis/100 mg. dry weight of suspension.)

Additions			
None	ATP (0·01 M)	Oxaloacetate (0·02 M)	Oxaloacetate + ATP
3·79	2·72	1·56	10·05
3·09	3·09	5·54	12·10
2·59	1·30	15·73	119·40
2·63	2·25	10·30	69·50
—	—	2·69	20·20
2·56	2·56	7·20	69·70

The gas phase used in the incubation had a great effect on fatty-acid synthesis and on the oxygen consumption of the suspensions. As can be seen from the results shown in Table 5, the amount of acetate incorporated into fatty acids was more than doubled when the gas phase was changed from pure oxygen to air. At the same time the $-Q_{O_2}$ increased from about 1·7–1·9 to 2·2–2·7. Incubation under nitrogen on the other hand caused an 80–90% inhibition of fatty-acid synthesis (Table 5; Expts. no. 83, 89). In the two experiments carried out under nitrogen the gas was first bubbled through the suspensions (kept in ice) for 15 min. and then, after the addition of the suspensions to the incubation flasks, the contents of the latter were equilibrated with nitrogen in the water bath for 10 min. It was thought that the slight but definite fatty-acid synthesis observed in these two experiments might have been due to a lack of completely anaerobic conditions. The experiment was therefore repeated in such a way that the suspension, after saturation with nitrogen, was incubated first with oxaloacetate for 15 min. and the acetate and ATP were then tipped in from the side arm. The results (not shown in the Table) were the same, i.e. only 90% inhibition of synthesis was observed. Citrate alone or in

Table 5. *Effect of gas phase and citrate on fatty-acid synthesis and on oxygen consumption of suspensions of rat mammary gland*

(Fatty acid synthesis expressed as μmoles $\times 10^{-3}$ of acetate incorporated into fatty acids/100 mg. dry weight of suspension. Final concentrations of additions were: oxaloacetate and citrate, 0·02 M; ATP, 0·01 M.)

		Gas phase				
		O_2		Air		N_2
Expt. no.	Additions	$-Q_{O_2}$	Fatty-acid synthesis	$-Q_{O_2}$	Fatty-acid synthesis	Fatty acid synthesis
83	(*a*) Oxaloacetate + ATP	1·67	36·7	2·22	91·5	11·2
	(*b*) Citrate	0·64	4·5	—	—	—
	(*c*) Citrate + ATP	1·97	5·3	—	—	—
89	Oxaloacetate + ATP	—	—	2·39	119·4	11·8
110	(*a*) Oxaloacetate + ATP	1·91	38·7	2·58	87·0	—
	(*b*) Oxaloacetate + ATP	1·86	43·5	2·49	97·0	—
	(*c*) Oxaloacetate + citrate + ATP	1·80	36·3	2·75	89·2	—
	(*d*) Oxaloacetate + citrate + ATP	1·93	31·7	2·50	84·6	—

combination with ATP was unable to stimulate fatty-acid synthesis (Table 5, Expt. no. 83); the amounts of ^{14}C incorporated into fatty acids by the suspensions in the presence of citrate were as low as were usually found when acetate or acetate and ATP were the only substrates added to the incubation medium. Furthermore, the addition of citrate to oxaloacetate and ATP did not affect the results obtained in the presence of the two last-named substances alone (Table 5, Expt. no. 110*c*, *d*). Experiment no. 110, which was carried out in duplicate, revealed also that the results with a particular suspension were reproducible to within about 10%.

α-Oxoglutarate, in contrast to oxaloacetate, stimulated fatty-acid synthesis to a significant degree even in the absence of added ATP; nevertheless ATP when added to α-oxoglutarate caused a further stimulation, particularly when the gas phase was air (Table 6). Pyruvate was also quite effective especially in combination with ATP, although the amount of acetate used for fatty-acid synthesis was not as great as in the presence of oxaloacetate and ATP (Table 7). As can be seen from the results shown in the same Table, succinate had no effect whatever on fatty-acid synthesis. Similarly negative results were obtained with malate and glutamate.

Since Balmain *et al.* (1952) have shown that glycerol stimulated fatty-acid synthesis from acetate in slices of rat mammary gland, glycerol was also tested in the suspensions, but either alone or in combination with other substances was found to have no effect on fatty-acid synthesis (Table 8).

Table 6. *Comparison of the effects of α-oxoglutarate and oxaloacetate on fatty-acid synthesis from* [carboxy-^{14}C]*acetate by suspensions of rat mammary gland*

(Results are expressed as μmoles $\times 10^{-3}$ of acetate used for fatty-acid synthesis/100 mg. dry weight of suspension. Final concentrations of additions were: oxaloacetate and α-oxoglutarate, 0·02 M; ATP, 0·01 M.)

Additions	Expt. no. 83 Gas phase, O_2	Expt. no. 89 Gas phase, air
None	—	2·54
ATP	—	1·30
Oxaloacetate	—	15·73
Oxaloacetate + ATP	36·7	119·40
α-Oxoglutarate	30·5	31·90
α-Oxoglutarate + ATP	34·6	74·70

Table 7. *Comparison of the effects of pyruvate and succinate with that of oxaloacetate on fatty-acid synthesis from* [carboxy-^{14}C]*acetate by suspensions of rat mammary gland*

(Results are expressed as μmoles $\times 10^{-3}$ acetate used for fatty-acid synthesis/100 mg. dry weight of suspension. Final concentrations of additions: oxaloacetate, pyruvate and succinate, 0·02 M; ATP, 0·01 M.)

Additions	Expt. no. 90
None	2·63
ATP	2·25
Oxaloacetate	10·30
Oxaloacetate + ATP	69·50
Pyruvate	18·40
Pyruvate + ATP	34·60
Succinate	3·11
Succinate + ATP	2·05

Table 8. *Comparison of the effect of glycerol and oxaloacetate on fatty-acid synthesis from* [carboxy-^{14}C]*acetate by suspensions of rat mammary gland*

(Fatty acid synthesis expressed as μmoles $\times 10^{-3}$ of acetate used/100 mg. dry weight of suspension. Expt. no. 93. Final concentrations of additions: ATP, 0·01 M; glycerol, 0·002 M; oxaloacetate, 0·02 M.)

Additions	Fatty acid synthesis
None	2·56
ATP	2·56
Glycerol	2·75
Glycerol + ATP	2·48
Oxaloacetate	7·20
Oxaloacetate + ATP	69·70
Oxaloacetate + glycerol + ATP	57·20

Table 9. *Fatty-acid synthesis from* [carboxy-^{14}C]-*acetate by suspensions of sheep udder*

(Results are expressed as μmoles $\times 10^{-3}$ of acetate used for fatty-acid synthesis/100 mg. dry weight of suspension. In (*a*), the suspension was prepared from fresh udder tissue; in (*b*) the suspension was prepared from the same udder tissue as in (*a*) but the tissue was kept frozen in solid CO_2 for 5 days. Final concentration of substrates, 0·02 M.)

Substrate added	Without ATP	With ATP (0·01 M)
Acetate (*a*)	69·4	10·2
Acetate (*b*)	46·8	6·8
Acetate + oxaloacetate (*a*)	111·0	62·3

Fatty-acid synthesis from [carboxy-^{14}C]*acetate by suspensions of sheep udder*

Suspensions of sheep udder, like slices of this tissue, can use acetate for fatty-acid synthesis very effectively without any further stimulant, although the addition of oxaloacetate to the medium nearly doubled the amount of acetate incorporated into fatty acids (Table 9). The amounts of acetate utilized by the sheep-udder suspension were very similar to those used by the suspensions of rat tissue in the presence of oxaloacetate and ATP. However, ATP in a concentration of 0·01 M acted in the sheep-udder suspension as a strong inhibitor of fatty-acid synthesis and of respiration also (see below). The suspension prepared from the udder tissue kept frozen in solid carbon dioxide for 5 days was almost as active as that made from the fresh udder in respect of fatty-acid synthesis.

Oxygen consumption of cell-free suspensions of rat mammary gland and of sheep udder

In every experiment the oxygen consumption of the suspensions was measured for the whole incubation period (3 hr.). The oxygen uptakes could not under all experimental conditions be related directly to fatty-acid synthesis. For example, usually the highest values of $-Q_{O_2}$ were observed when succinate was added to the suspensions, no doubt on account of the high succinic dehydrogenase activity of the preparations (cf. Moore & Nelson, 1952), yet no significant fatty-acid synthesis occurred. For this reason we wish to record only some of the results which illustrate the different responses of the suspensions made from the rat and sheep tissues to ATP (Figs. 1 and 2). In the suspensions of rat mammary gland the effect of ATP was to increase very significantly the oxygen uptake, whereas in the sheep-udder suspension ATP caused a dramatic inhibition of oxygen uptake. We do not think that the simultaneous inhibition of fatty-acid synthesis by ATP in sheep-udder suspension (Table 9) is necessarily connected with the lowered respiration of the preparation because, as can be seen from Fig. 2, oxaloacetate had an even greater inhibiting effect on the oxygen consumption than ATP yet it nearly doubled the amount of acetate incorporated into fatty acids (Table 9).

The addition of acetate (0·02 M) in all suspensions, from either rat or sheep tissues, invariably depressed the oxygen consumption of the preparations. This phenomenon again does not reflect the ability or inability of the suspensions to oxidize acetate. Suspensions of rat mammary gland oxidized only traces of acetate, whereas the preparation from the fresh sheep udder oxidized 0·19 μmoles of acetate/100 mg. dry weight, but in the presence of ATP (0·01 M) only 0·06 μmoles of acetate were oxidized/100 mg. dry weight. Oxaloacetate diminished the amount of acetate oxidized by the suspension of sheep udder to an almost negligible level, an effect qualitatively similar to that observed in slices.

Type of fatty acids synthesized by suspensions of rat mammary gland

The fatty acids extracted from four rat mammary suspensions after incubation with [*carboxy*-^{14}C]-acetate in the presence of oxaloacetate and ATP were pooled and resolved into individual acids by chromatography as described under Methods. The results are shown in Table 10. All the saturated *n*-fatty acids containing an even number of carbon atoms from 6 to 18 and also oleic acid were obtained. We suspect that the small amount of butyric acid, which we expected to find, was lost during the various manipulations owing to the solubility in water and volatility of this acid. Since all the acids were radioactive it may be concluded that the preparations synthesize both short- and long-chain fatty acids. The figures in the last column of Table 10 show that most of the ^{14}C found in the fatty acids (about 91 %) could be accounted for by four fractions, namely, caproic, myristic, palmitic and oleic acids. It can be seen also from the data of Table 10

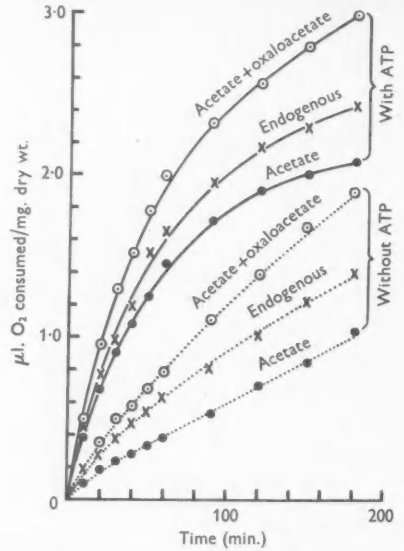

Fig. 1. The effect of substrates and ATP on O_2 consumption of rat mammary-gland suspensions. The additions were as indicated over the curves.

Table 10. *Type of fatty acids synthesized from* [carboxy-^{14}C]*acetate by suspensions of rat mammary gland in the presence of oxaloacetate and ATP*

Fatty acid	Specific radioactivity (counts/min./C in infinite thickness)	Fatty-acid fraction as % of total	^{14}C recovered in fraction as % of total
Hexanoic	6060	3·72	24·00
Octanoic	603	1·60	1·12
Decanoic	325	5·75	2·27
Dodecanoic	657	6·20	4·92
Tetradecanoic	925	11·60	13·62
Hexadecanoic	960	28·80	35·63
Octadecanoic	108	7·38	0·82
Octadec-9-enoic	382	35·00	17·62

that the fatty acids were not synthesized strictly in the proportions in which they occurred in the suspensions.

DISCUSSION

The work of Brady & Gurin (1952*a*, *b*), Dituri & Gurin (1953) and the results presented here demonstrate that the integrated structure of the cell is not required for fatty-acid synthesis. Whether or not a net increase occurred in the amount of fatty acids in our preparation, or in those of the American workers, cannot be decided at present. The measurement of the respiratory quotients of suspensions might have given this information (cf. Folley, 1952).

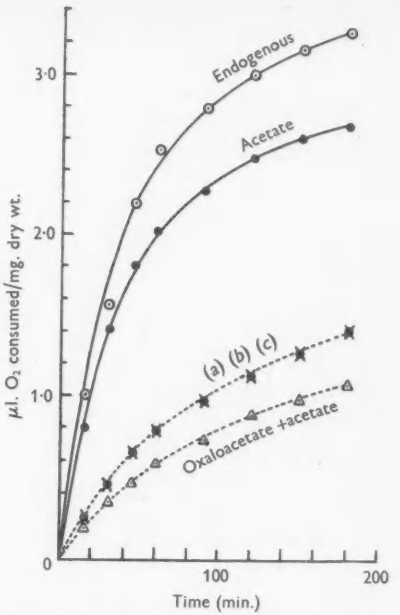

Fig. 2. The effects of acetate, oxaloacetate and ATP on O_2 consumption of a sheep-udder suspension. Curve marked (*a*) (*b*) (*c*) indicates the O_2 consumption under three experimental conditions, namely (*a*) ATP alone; (*b*) acetate + ATP; and (*c*) acetate + oxaloacetate + ATP.

Our experiments with the suspensions of rat mammary gland show that three conditions are required in this system for fatty-acid synthesis from acetate: (*a*) aerobic incubation; (*b*) the oxidation of either of the three keto acids, pyruvate, oxaloacetate or α-oxoglutarate and (*c*) the addition of ATP, although strictly the latter is not necessary with pyruvate or α-oxoglutarate. In suspensions of sheep udder neither the addition of the keto acids nor of ATP is required for fatty-acid synthesis from acetate, although oxaloacetate had a stimulating effect. The species differences in response to ATP are at present intriguing. Brady & Gurin (1952*b*) reported that in suspensions of pigeon liver and in a soluble enzyme preparation made from it ATP had either no effect on fatty-acid synthesis or in concentrations greater than 1×10^{-3} M was inhibitory. There appear to be some significant differences between the mammary gland preparations and those made from pigeon liver. According to Brady & Gurin (1952*b*) the maximum stimulation of fatty-acid synthesis in the pigeon-liver preparation was observed in the presence of citrate, which in our system had no effect whatever; oxaloacetate, which showed the greatest stimulation in the mammary-

gland suspensions, had but a slight effect in the pigeon-liver suspensions. (It should, however, be pointed out that in their preliminary communication Brady & Gurin (1952*a*) reported that oxaloacetate had the greatest stimulating effect on fatty-acid synthesis in the pigeon-liver suspensions and that citrate was inhibitory.)

A further significant difference is that whereas in the suspensions of mammary gland incubation under nitrogen caused an 80–90% inhibition of fatty-acid synthesis, and therefore the energy required for the synthesis presumably has been derived from the oxidation of substrates, in the pigeon-liver preparations anaerobic conditions had only a slight inhibitory effect or none at all. Although these differences seem to be great, it is not likely that they are due to differences in the principal reactions of biosynthesis of fatty acids in the two systems; it seems more probable that they reflect qualitative and quantitative differences in the preparations in respect of the enzymes required for the activation of acetate.

The reason for the very striking effect of the gas phase, namely, the increased fatty-acid synthesis and higher oxygen consumption of the suspensions when the gas phase was changed from pure oxygen to air, is at present obscure. It is possible that, under high oxygen tension, oxidation of —SH groups occurred, resulting in an inactivation of enzymes and/or coenzymes.

The species differences in the metabolism of the mammary gland, which were elucidated by Folley and his colleagues (see Folley, 1952) in experiments with tissue slices, are apparent even in the suspensions. For example, while suspensions of rat mammary gland are virtually unable to oxidize acetate, preparations from sheep udder were fairly efficient in this respect. It seems probable that in the mammary gland of the rodent there are blocks in the reactions of the citric acid cycle, which do not exist in the tissues of the ruminant. Such an assumption might explain why the sheep-udder preparation does not require the addition of ATP. On the other hand we cannot yet offer a satisfactory explanation for the marked inhibition of fatty-acid synthesis, of acetate oxidation and of oxygen consumption caused by ATP in the sheep-udder suspensions.

The value of the mammary-gland preparations in the study of the biosynthesis of fatty acids seems to us to lie in their ability to synthesize fatty acids of all chain lengths. The fact that the fatty acids in the suspensions of rat mammary gland were not synthesized strictly in the proportions in which they were present suggests that different enzymes might be involved in the synthesis of groups of acids of varying chain lengths. It is of interest to mention here that Green & Mii (1953) reported that in the oxidation of fatty acids of different chain lengths from C_4 to C_{18} at least three enzymes covering specific ranges of the acids are involved. The stepwise increase in the specific activity of the fatty acids from C_{10} to C_{16} (cf. Table 10) resembles the findings in the milk fatty acids of the lactating goat (Popják, French, Hunter & Martin, 1951) in which it was shown that such a stepwise increase in the specific activity of the acids after injection of [*carboxy*-^{14}C]acetate was due to the stepwise elongation of a shorter acid to a longer one.

SUMMARY

1. The biosynthesis of fatty acids from acetate in tissue slices and cell-free suspensions of rat and sheep mammary gland have been studied.

2. In tissue slices, in addition to glucose, pyruvate and members of the citric acid cycle stimulated fatty-acid synthesis from acetate. These substances were particularly effective with slices of sheep udder.

3. Cell-free suspensions of the mammary gland of lactating rats and sheep can synthesize fatty acids from [*carboxy*-^{14}C]acetate.

4. In suspensions of rat mammary gland three conditions are required for fatty-acid synthesis: (*a*) aerobic incubation; (*b*) the oxidation of either pyruvate, oxaloacetate or α-oxoglutarate; and (*c*) the addition of adenosine triphosphate to some extent. The best results were obtained in the presence of oxaloacetate and adenosine triphosphate and when the gas phase was air instead of pure oxygen.

5. All the saturated *n*-fatty acids containing an even number of carbon atoms from 6 to 18 and also oleic acid were synthesized in the rat mammary suspensions.

6. Suspensions of sheep udder used acetate for fatty-acid synthesis even without further addition, although oxaloacetate had a stimulating effect in this preparation also, but adenosine triphosphate in a concentration of 0·01 M inhibited fatty-acid synthesis.

7. While the suspension of sheep udder oxidized appreciable amounts of acetate, suspensions of rat mammary gland were virtually inactive in this respect.

8. The implications of the results are discussed.

The technical assistance of Miss Mary O'Donnell is gratefully acknowledged.

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Paper-chromatographic Analysis of Dakin's Samples of 'β-Hydroxyglutamic Acid'

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(*Received* 1 *May* 1953)

Dakin twice claimed the isolation from proteins of β-hydroxyglutamic acid. In his first paper (Dakin, 1918) he obtained a 10·5% yield but later (Dakin, 1919) he reported more variable yields of 2–10%. The compound had been obtained by acid and by tryptic digestion of casein and also by acid hydrolysis of gliadin and glutenin. Many workers have been unable to repeat this work and doubt is now generally cast on its validity (Nicolet & Shinn, 1942; Bailey, Chibnall, Rees & Williams, 1943; Beveridge & Lucas, 1944). It is of note that Dakin himself in a footnote to one of his papers (Dakin, 1941) commented on the fact that one reaction of his amino acid was inconsistent with its structure as a β-hydroxy-α-amino acid and that he was therefore exploring further the possibility that it was a γ-hydroxy compound. The amino acid still, however, continues to occupy space in the standard textbooks, since, using classical methods of analysis and isolation, it is difficult to be certain of the absence from a protein of such a substance. The only certain way to assess the earlier work is to re-examine the original samples in question. Dr H. B. Vickery has now kindly obtained for us two samples of β-hydroxyglutamic acid from Dakin's collection. These we have analysed by the simpler and far more specific methods now available.

MATERIALS

Two of Dakin's specimens were obtained from the Dakin Collection, Adelphi College. They were labelled: (1) 'Hydroxyglutamic acid *ex* casein and trypsin.' (2) 'Hydroxy-glutamic acid, analysed.' (1) was a mixture of brown particles and powder, (2) comprised 1 mg. only of brown particles. They will be referred to here as Dakin's specimens nos. 1 and 2, respectively.

For comparison with Dakin's samples, synthetic specimens of DL-β-hydroxyglutamic and DL-*allo*-β-hydroxyglutamic acids were obtained by Dr Vickery from Dr Pfister. Both substances were white crystalline powders. The methods of synthesis and the convention of nomenclature have been described by Leanza & Pfister (1953).

METHODS

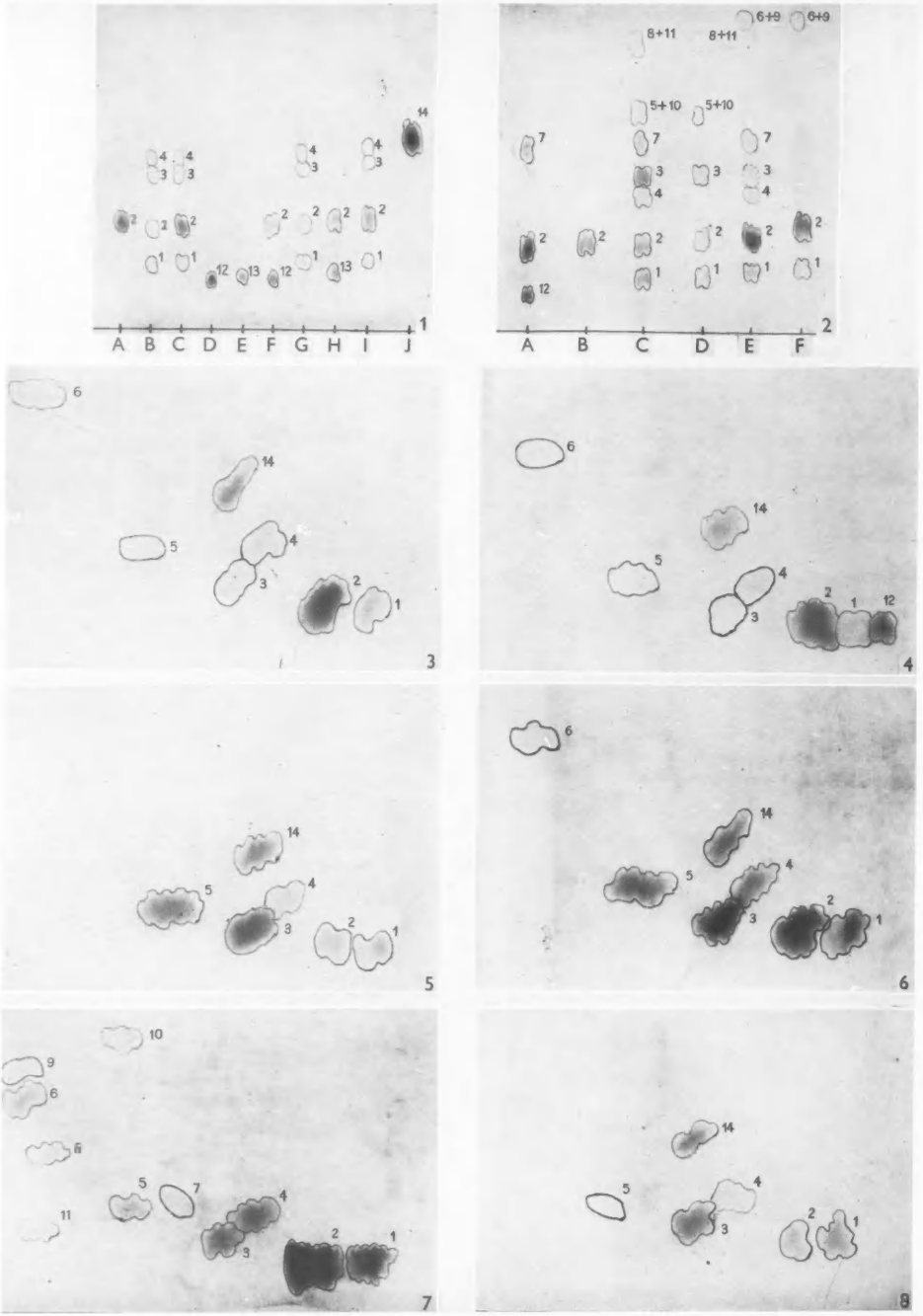
The analyses were performed on 0·1% (w/v) solutions of the amino acids in 10% (v/v) aqueous *iso*propanol. The two Dakin specimens dissolved immediately in the solvent, whereas the two synthetic compounds only dissolved slowly after much shaking. The amino acids used as markers were in the same solvent at a concentration of 0·01 M.

One-way paper chromatograms were run with phenol:water in the presence of NH_3 and HCN. Two-way chromatograms were run with this system first followed by collidine:lutidine:water in the presence of diethylamine vapour. The apparatus and working details were as described by Dent (1948). Both the synthetic isomers of β-hydroxyglutamic acid gave brownish purple spots on the chromatogram which could be readily distinguished from the adjacent spots given by other amino acids.

Periodate treatment of the amino acids was carried out by adding about 2 vol. of saturated aqueous KIO_4 and 0·5 vol. of saturated aqueous Na_2CO_3 to 1 vol. of amino acid solution. The mixture was allowed to stand for 0·5 hr. and was then electrolytically desalted in a micromodification (Dent, 1952) of the apparatus of Consden, Gordon & Martin (1947).

RESULTS

Chromatograms of the various samples compared with authentic amino acids were prepared as shown on Pl. 1. They were prepared with the intention of showing whether Dakin's samples contained any β-hydroxyglutamic acids, whether they contained any other β-hydroxyamino acids or any of the commonly occurring amino acids, and if so which. The conclusions drawn from the analyses are written into the description of the Plate (p. 55).



C. E. DENT AND DOROTHY I. FOWLER—PAPER-CHROMATOGRAPHIC ANALYSIS OF DAKIN'S SAMPLES OF 'β-HYDROXYGLUTAMIC ACID'

SUMMARY

1. Two samples of material isolated from protein hydrolysates by the late H. D. Dakin, and alleged by him to be β-hydroxyglutamic acid, consist of mixtures of commonly occurring amino acids, mainly of glutamic and aspartic acids.

2. No β-hydroxyglutamic acid was present in either sample.

We wish to thank Dr H. B. Vickery for his initiative in stimulating this work and for obtaining the samples for us.

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EXPLANATION OF PLATE 1

All the chromatograms were run by the descending technique. The one-way chromatograms shown on Figs. 1 and 2 were run with phenol-water: NH_3: HCN (shown in an upward direction), the amino acids being applied on the base line as marked. The two-way chromatograms (Figs. 3–8) were run with phenol-water: NH_3: HCN as first solvent (shown right to left) followed by collidine: lutidine: water: diethylamine (shown in an upward direction); the origin is near the plate number.

The spot identifications are as follows: 1, aspartic acid; 2, glutamic acid; 3, glycine; 4, serine; 5, alanine; 6, leucine and/or isoleucine; 7, threonine; 8, valine; 9, phenylalanine; 10, tyrosine; 11, lysine; 12, β-hydroxyglutamic acid; 13, *allo*-β-hydroxyglutamic acid; 14, taurine.

FIG. 1

One-way chromatogram. Solutions of the following substances were placed on the correspondingly marked starting positions: A, L-glutamic acid (5 μl.); B, Dakin's specimen no. 1 (10 μl.); C, Dakin's specimen no. 2 (10 μl.); D, DL-β-hydroxyglutamic acid (5 μl.); E, DL-*allo*-β-hydroxyglutamic acid (5 μl.); F, glutamic (5 μl.) and β-hydroxyglutamic (5 μl.) acids; G, Dakin's specimen no. 1 (10 μl.); H, glutamic (5 μl.) and *allo*-β-hydroxyglutamic (5 μl.) acids; I, Dakin's specimen no. 2 (10 μl.); J, taurine (50 μl.). The numbers indicate the provisional identifications of the spots. It was concluded that neither of Dakin's specimens contain an amino acid behaving like a β-hydroxyglutamic acid.

FIG. 2

One-way chromatogram. Solutions of the following substances were placed on the correspondingly marked starting positions with additional pretreatment as indicated: A, L-glutamic (5 μl.) and DL-β-hydroxyglutamic (5 μl.) acids and DL-threonine (5 μl.); B, glutamic (15 μl.) and β-hydroxyglutamic (15 μl.) acids and threonine (15 μl.), periodate-treated; C, Dakin's specimen no. 1 (20 μl.) and threonine (5 μl.); D, Dakin's specimen no. 1 (20 μl.) and threonine (5 μl.), periodate-treated; E, Dakin's specimen no. 2 (20 μl.) and threonine (5 μl.); F, Dakin's specimen no. 2 (20 μl.) and threonine (5 μl.), periodate-treated. The numbers indicate the provisional identifications of the spots. It was concluded that the amino acids in Dakin's specimens which behave chromatographically somewhat like synthetic β-hydroxyglutamic acids are not decomposed by periodate and are therefore not β-hydroxy-α-amino acids. Only one amino acid originally in Dakin's specimen is so decomposed; this is presumably serine.

FIG. 3

Two-way chromatogram of Dakin's specimen no. 2 (50 μg.) with taurine (10 μl.) added as a marker. The numbers indicate the fairly definite identifications that could now be made. It was concluded that this specimen contains glutamic acid with lesser amounts of other known amino acids.

FIG. 4

As Fig. 3, but with DL-β-hydroxyglutamic acid (10 μl.) added. It was concluded that β-hydroxyglutamic acid can be distinguished on our chromatograms from the main amino acids present in Dakin's specimen no. 2.

FIG. 5

Two-way chromatogram of L-aspartic and L-glutamic acids, glycine, DL-serine, DL-alanine and taurine (10 μl. of each). This should be compared with the chromatograms of Dakin's specimens no. 1 (Fig. 8) and no. 2 (Fig. 3).

FIG. 6

Two-way chromatogram of Dakin's specimen no. 2 (50 μg.) and the same pure amino acids as for Fig. 5 (10 μl. of each). The pure amino acids run to exactly the same positions as do those in Dakin's specimen no. 2.

FIG. 7

Two-way chromatogram of Dakin's specimen no. 2 (250 μg.). Even at this overload the amino acid spots shown appear to correspond with known substances, and no spot is seen in the position occupied by β-hydroxyglutamic acid.

FIG. 8

Two-way chromatogram of Dakin's specimen no. 1 (50 μg.) with taurine (10 μl.) added. It was concluded that this specimen comprises substantially the same amino acids as no. 2 (Fig. 3) but in different relative proportions.

Studies in the Biochemistry of Micro-organisms

91. THE COLOURING MATTERS OF *PENICILLIUM ISLANDICUM* SOPP. PART 3. SKYRIN AND FLAVOSKYRIN

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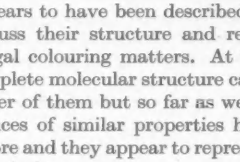
(*Received* 11 *July* 1953)

In the first communication from this laboratory dealing with the colouring matters of *Penicillium islandicum* Sopp (Howard & Raistrick, 1949), it was shown that five out of the six strains of this species which were examined produced a hitherto undescribed colouring matter, islandicin, which was proved, by analytical methods, to be 1:4:5-trihydroxy-2-methylanthraquinone (I). This conclusion was confirmed by Joshi, Tilak & Venkataraman (1950) who prepared the compound by an unambiguous synthesis and showed that it was identical with natural islandicin. Re-examination of the sixth strain of this species (Howard & Raistrick, 1950), which bears the catalogue number N.R.R.L. 1175 and which is much more orange and less red in colour than the other five strains, failed to reveal any islandicin even when its mixed colouring matters were submitted to chromatographic analysis. It was shown however that the complex mixture of colouring matters present in the dried mycelium of this strain contained chrysophanol (chrysophanic acid) (II) and two other crystalline colouring matters which were not named or described at that time. It is the purpose of the present communication to describe the isolation and properties of these two substances, neither of which appears to have been described previously, and to discuss their structure and relationship to other fungal colouring matters. At the present time, a complete molecular structure cannot be assigned to either of them but so far as we are aware, no substances of similar properties have been described before and they appear to represent completely new chemical types:

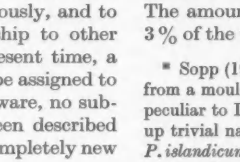
When the dried and ground mycelium of the mould, a brown powder, was extracted with benzene at room temperature, a dark orange solution was obtained which contained numerous pigments. This solution was passed through columns of 'heavy' magnesium carbonate, and chrysophanol was readily isolated from the eluate. Development of the chromatograms with methanol produced *inter alia* an ochre-yellow band, from which was obtained a substance crystallizing as yellow needles, m.p. 215° (decomp.). We propose for it the name flavoskyrin* and shall describe it more fully later.

Further extraction of the mycelial powder with hot benzene in a Soxhlet apparatus removed a mixture of pigments similar in composition to that obtained with cold benzene, together with another substance which separated as a dark-brown powder on standing. This substance which when pure is dark-orange or yellow in colour, does not melt below 380°. We propose to call it skyrin. Further quantities of chrysophanol and flavoskyrin were isolated, as described above, from the hot benzene Soxhlet extracts after removal of crude skyrin, and further quantities of skyrin were obtained by subsequent exhaustive extraction of the mycelium with acetone. The amount of purified skyrin isolated was about 3 % of the weight of the dried mould mycelium but

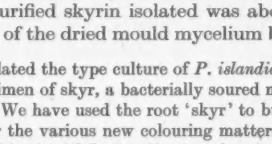
* Sopp (1912) isolated the type culture of *P. islandicum* from a mouldy specimen of skyr, a bacterially soured milk peculiar to Iceland. We have used the root 'skyr' to build up trivial names for the various new colouring matters of *P. islandicum*, qualifying it with Latin adjectives descriptive of the colour or appearance of the pigment concerned.



(I)
Islandicin



(II)
Chrysophanol



(III)
Emodin

the total amount present in it is undoubtedly much greater. The yields of flavoskyrin varied between 0·2 and 0·7 %.

Skyrin

Skyrin, $C_{30}H_{18}O_{10}$, is dimorphic, crystallizing either as clusters of dark-orange, pointed rods or as hexagonal, yellow plates. Both forms darken but do not melt when heated to 380°. The molecule contains six hydroxyl groups which can be acetylated or methylated, two quinone groupings and two methyl groups attached to carbon. It contains no methoxyl groups. It is insoluble in 0·5N sodium bicarbonate but dissolves in N ammonia, N sodium carbonate and N sodium hydroxide to deep-purple coloured solutions. Skyrin exhibits very characteristic reactions with cold concentrated sulphuric acid, the two crystalline forms differing slightly in their behaviour towards this reagent. The orange crystals dissolve to a deep cherry-red solution, which immediately begins to turn brown and finally changes to a stable, emerald-green solution, the whole change taking about one minute. The yellow crystals dissolve to give the emerald-green solution immediately without the preliminary cherry-red colour. One of other of these two reactions with cold concentrated sulphuric acid is shown by all the derivatives of skyrin which have been prepared with the exception of the hexamethyl ether. The yellow form of the substance can be converted into the orange form by dissolving in alkali and acidifying, when orange material is precipitated which gives the red to green reaction in concentrated sulphuric acid. The green sulphuric acid solutions prepared from either form of skyrin yield material of the orange form when diluted with water. The two forms seem readily interconvertible in solution, but not in the solid phase.

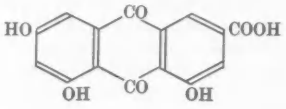
The following functional derivatives of skyrin have been prepared. (*a*) Hexaacetylskyrin, $C_{42}H_{30}O_{16}$, i.e. $C_{30}H_{12}O_4(O.CO.CH_3)_6$, by heating with acetic anhydride and anhydrous sodium acetate. It forms thin yellow needles, m.p. 295–296°, and is optically inactive in chloroform solution. It is insoluble in 0·5N sodium bicarbonate and in N sodium carbonate, and is slowly soluble, with hydrolysis to skyrin, in cold aqueous N sodium hydroxide. (*b*) Skyrin hexamethyl ether, $C_{36}H_{30}O_{10}$, i.e. $C_{30}H_{12}O_4(OCH_3)_6$, by heating with dimethyl sulphate and anhydrous potassium carbonate in acetone solution. It forms an orange microcrystalline powder on sublimation in a high vacuum at 300–310° and melts at 370–372°. It is insoluble in alkalis; its solution in cold concentrated sulphuric acid is a stable, red-purple colour, which is indistinguishable from that of a solution, in the same reagent, of emodin trimethyl ether (i.e. the trimethyl ether of III). (*c*) Skyrin pyridine salt, $C_{40}H_{28}O_{10}N_2$, i.e. $C_{30}H_{18}O_{10}$, $2C_5H_5N$, by crystallization from pyridine. It forms bright red, rhombohedral crystals which blacken without melting on heating.

Skyrin forms compounds with methyl and ethyl alcohols, either (*a*) on long standing in the alcoholic solution or, more rapidly, (*b*) by boiling a solution of hexaacetylskyrin in the alcohol containing concentrated sulphuric acid. Dimethylskyrin, $C_{32}H_{22}O_{10}$, i.e. $C_{30}H_{16}O_8(OCH_3)_2$, prepared by method (*b*) forms orange-yellow plates which do not melt below 360°. Diethylskyrin, $C_{34}H_{26}O_{10}$, i.e. $C_{30}H_{16}O_8(OC_2H_5)_2$, prepared by both methods (*a*) and (*b*) forms yellow plates from dioxan which do not melt below 360°. Unlike skyrin itself, both derivatives are insoluble in N sodium carbonate. Both dissolve in cold aqueous N sodium hydroxide to orange solutions which slowly become purple probably due to hydrolysis to skyrin. Both dissolve in cold concentrated sulphuric acid to give a green solution immediately. The nature of these two substances is not absolutely clear and must be left unsettled for the present. Despite their method of preparation they cannot be dimethyl or diethyl esters since skyrin does not contain any carboxyl groups. Both substances have some properties which suggest that they may be β-methyl ethers of a polyhydroxyanthraquinone, e.g. their insolubility in N sodium carbonate, although skyrin is readily soluble in this reagent. Typical β-methyl ethers of substituted anthraquinones, e.g. physcion, 4:5-dihydroxy-7-methoxy-2-methylanthraquinone, i.e. 7-methyl ether of (III), cannot however be prepared from the parent substance, except in extremely minute yields, by either method (*a*) or (*b*) above and are not hydrolysed to the parent substance by cold aqueous N sodium hydroxide.

Oxidation with chromic acid of hexaacetylskyrin in acetic anhydride-acetic acid solution results in the conversion of the two *C*-methyl groups into two COOH groups and the formation of the dibasic hexaacetylskyric acid, $C_{42}H_{26}O_{20}$, i.e. $C_{30}H_8O_8(O.CO.CH_3)_6$ as yellow, oblique-ended rods, m.p. 231–232° (decomp.). Skyric acid, $C_{30}H_{14}O_{14}$, $3H_2O$, was prepared by alkaline hydrolysis of hexaacetylskyric acid and was obtained as bright orange-red rods which do not melt below 370°. Skyric acid, like skyrin, exists in two forms which show the same characteristic differences in their reactions with cold concentrated sulphuric acid. When a methanolic solution of hexaacetylskyric acid, containing concentrated sulphuric acid, was boiled, the dimethyl ester of skyric acid (dimethyl skyrate), $C_{32}H_{18}O_{14}$, i.e. $C_{28}H_{12}O_{10}(COOCH_3)_2$, was obtained as yellow, pointed rods which do not melt below 370°.

Our clearest insight into the molecular structure of skyrin was provided by the surprising discovery that when a cold alkaline aqueous solution of skyrin

is treated with sodium dithionite ($Na_2S_2O_4$) it rapidly undergoes reductive fission with more than a 90% quantitative formation of two molecules of *Frangula* emodin (III) according to the equation $C_{30}H_{18}O_{10} + 2H \rightarrow 2C_{15}H_{10}O_5$. This observation was reported by one of us in the Bakerian Lecture of the Royal Society (Raistrick, 1949). It was found later that skyric acid on similar treatment is converted, though not quite so smoothly, into emodic acid (IV):

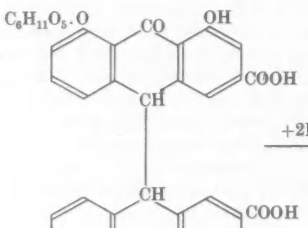


(IV)

Emodic acid

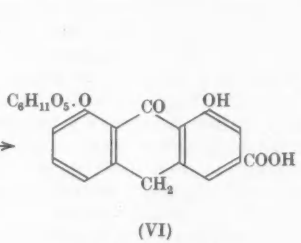
This experimental evidence is explained most logically by postulating that the molecule of skyrin consists of two emodin molecules linked together in such a way that both quinone groupings are intact and the six hydroxyl groups and two *C*-methyl groups are free. This inevitably means that the two molecules of emodin are joined in skyrin by a carbon-carbon linkage, and that this linkage is broken almost quantitatively by treatment with sodium dithionite in cold, alkaline aqueous solution, an assumption that seemed highly improbable at the time when the experimental observation was made and reported on. However, Stoll, Becker & Helfenstein (1950), in one of a series of communications on sennosides A and B, the active glycosides of the laxative senna drugs of commerce, showed that these substances and their corresponding aglycones, the sennidins A and B, undergo reductive cleavage by catalytic hydrogenation in the presence of palladium at room temperature or, even better, by treatment of the esters of the glycosides or the salts of the aglycones with sodium dithionite.

In a review article by Stoll & Becker (1950) on 'Sennosides A and B, the Active Principles of Senna', the evidence, including synthetic evidence, is assembled on which they advance structure (V) for the stereoisomeric sennosides A and B and structure (VI) for the product of reductive fission which, as will be seen is derived from the substituted anthraquinone, rhein (VII).



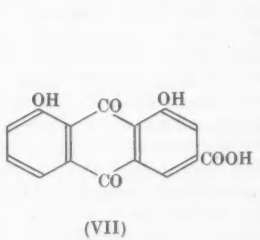
(V)

Sennosides A and B



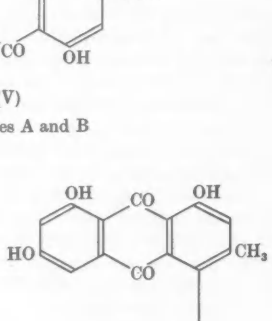
(VI)

8-Glucosido-1:8-dihydroxyanthrone-3-carboxylic acid

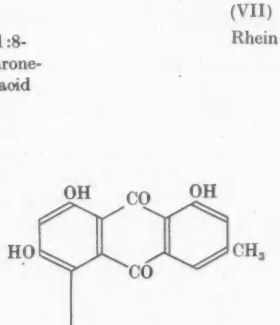


(VII)

Rhein



(VIII)



(IX)

It is clear from this evidence that, under certain conditions, the carbon-carbon linkage present in the substituted dianthrones, the sennosides A and B, can be readily broken by sodium dithionite. Assuming, as seems probable, that a carbon-carbon linkage in a substituted dianthraquinone can be broken by the same reagent, the problem of the constitution of skyrin resolves itself now into the difficult decision as to which of the free positions 1, 3, 6, 8 in each of the two molecules of emodin are joined together in the skyrin molecule. We have no experimental evidence available to decide this point so that, of the possible structures for skyrin, (VIII) and (IX) are advanced merely to indicate the type of structure which we believe skyrin to possess.

Either of these structures offers a satisfactory explanation of many of the properties of skyrin which we have recorded. Thus, the initial red colour of a solution of skyrin in cold concentrated sulphuric acid is very similar to that given by emodin itself, and it may well be that the final emerald-green colour of such skyrin solutions results from interaction between hydroxyl groups on the two emodin nuclei. In the case of the hexamethyl ether of skyrin, where such interaction is impossible, the sulphuric acid solution is indistinguishable in colour from that given by the trimethyl ether of emodin. The presence of two β-hydroxyl groups in the molecule of skyrin, one in each emodin moiety, explains the ready solubility of skyrin in aqueous N sodium carbonate, and also the formation of a pyridine salt containing two molecules of pyridine. The two β-hydroxyl groups also seem to be involved in the formation of the dimethyl and diethyl derivatives of skyrin described on p. 62, since these compounds are insoluble in aqueous N sodium carbonate. Finally, the preparations of hexaacetylskyric acid and of dimethyl skyrate are exactly analogous to the oxidation of triacetylemodin to triacetylemodic acid by Fischer & Gross (1911) and the conversion of the latter substance into methyl emodate by Anslow, Breen & Raistrick (1940). Skyric acid, in which the two CH_3 groups present in skyrin are oxidized to two COOH groups, may thus be considered to be the emodic acid analogue of skyrin.

A number of mould colouring matters, having high melting points, are almost certainly polyhydroxydianthraquinones and some of them are clearly derivatives of emodin. Penicilliopsin, from the mould *Penicilliopsis clavariaeformis* Solms-Laubach (Oxford & Raistrick, 1940), has the empirical formula $C_{30}H_{24}O_8$ and melts at about 330°. On thermal decomposition it yields emodin anthranol, and on oxidation with nitric acid it gives tetranitroemodin. Aurofusarin, from *Fusarium culmorum* (W. G. Smith) Sacc. (Ashley, Hobbs & Raistrick, 1937), has the empirical formula $C_{30}H_{20}O_{12}$ and does not melt below 360°. Little is known of its constitution except that it contains two methoxyl groups, and that its general properties are those of a polyhydroxydianthraquinone.

Flavoskyrin

Flavoskyrin, the less abundant of the two colouring matters described in this communication, crystallizes readily from dioxan and acetic acid or from acetone and water, forming light-yellow needles, m.p. 215° (decomp.). Its molecular formula is probably $C_{15}H_{12}O_5$, though this is suggested somewhat tentatively since it has not yet been possible to prepare a crystalline derivative for confirmation. Flavoskyrin is insoluble in 0·5 N sodium bicarbonate but in N sodium carbonate and in N sodium hydroxide it dissolves to a yellow solution, the colour of which is similar to that of a solution of the free substance in organic solvents. A bright-yellow, uniform, stable band is formed when a solution is passed through a column of magnesium carbonate. The colour of the substance is not discharged either by sodium dithionite ($Na_2S_2O_4$) or by zinc and acetic acid.

When heated to its melting point flavoskyrin decomposes almost quantitatively into chrysophanol (II) and water according to the equation $C_{15}H_{12}O_5 \rightarrow C_{15}H_{10}O_4 + H_2O$. Chrysophanol is also formed, but in slightly lower yield, when flavoskyrin is dissolved in cold concentrated sulphuric acid; the solution is at first bright orange and changes during 2–3 hr. to a purple-red, as decomposition proceeds. Flavoskyrin was acetylated with acetic anhydride and pyridine at room temperature and was methylated with dimethyl sulphate and potassium carbonate in boiling acetone. Neither of the crude products so obtained could be crystallized.

In the present state of our knowledge speculation regarding the structure of flavoskyrin would be unprofitable. We believe however that, from its properties recorded above, of the many possibilities, it is improbable that flavoskyrin is either a substituted naphthaquinone, benzoylbenzoic acid or anthrahydroquinone. It is hoped to communicate further experimental evidence leading to a solution of the problem at a later date.

Chrysophanol

The facts that flavoskyrin is readily converted into chrysophanol and that both these substances have been isolated from the mycelium of *P. islandicum*, strain N.R.R.L. 1175, suggest the possibility that chrysophanol may be an artifact arising from flavoskyrin during the process of isolation, e.g. (*a*) during drying of the mycelium *in vacuo* at 40–42°. This possibility was disproved as follows. Fresh mycelium, without drying, was disintegrated at room temperature in a high-speed macerater in the

presence of benzene. Chrysophanol was found in the benzene extract. (*b*) During Soxhlet extraction of the dried mycelium with boiling benzene or boiling light petroleum, b.p. 40–60°. This possibility was disproved by the observation that chrysophanol was present in a benzene extract of the dried mycelium made at room temperature. (*c*) During chromatography on magnesium carbonate columns of the various solvent extracts. This was disproved by chromatographing pure flavoskyrin. A single, yellow band was obtained on magnesium carbonate, and prolonged washing with either benzene or methanol did not lead to the formation of a pink band below it as would happen if chrysophanol were being formed.

We consider therefore that at least part of the chrysophanol extracted from the mycelium is a true metabolic product of *P. islandicum*, strain N.R.R.L. 1175.

EXPERIMENTAL

Culture

The strain of *Penicillium islandicum* Sopp used in this work was received in December 1947 from the Thom-Raper collection from Dr Kenneth B. Raper, Northern Regional Research Laboratory (N.R.R.L.), U.S. Department of Agriculture, Peoria, Illinois, U.S.A., and bears his catalogue number N.R.R.L. 1175. It is the same strain as that from which chrysophanol was obtained by Howard & Raistrick (1950). Mature cultures of this strain are much more orange and less red in colour than a number of other strains of *P. islandicum* received from Dr Raper.

Cultural conditions

Culture medium. Czapek-Dox solution. Glucose, 50·0 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4$, $7H_2O$, 0·5 g.; $FeSO_4$, $7H_2O$, 0·01 g.; distilled water, 1 l. Batches of 100 one-litre conical flasks, each containing 350 ml. of Czapek-Dox solution, were sterilized and inoculated with *P. islandicum*, strain N.R.R.L. 1175. Details of inoculation, incubation and harvesting of the mycelium were the same as those previously described (Howard & Raistrick, 1950). After 20–24 days' incubation at 24° the glucose content of the medium had fallen to 0·3 % or less and the pH was about 4·1. The yield of dry mycelium was about 3·2 g./flask.

Isolation of the pigments

The dried and ground mycelium from a batch of 100 flasks, 320 g., was stirred with benzene (3·2 l.) at room temperature during 2 working days. The extract was separated by filtration from the mycelium which was then submitted to a second similar extraction. Tests on the twice-extracted mycelium showed it to contain only a negligible amount of chrysophanol.

The dark-orange benzene extracts were chromatographed on columns of heavy $MgCO_3$. About 250 ml. of the first extract or 750 ml. of the second extract were passed through each column, 20 × 2·5 cm., followed by 100 ml. of fresh benzene. The mixture of pigments split into several bands which were, from top to bottom: dark-orange, narrow orange-red, broad yellow, narrow brown, pale purple and pale salmon-pink. The two extracts gave similar chromatograms, except that the second gave a much narrower yellow band. On washing with benzene the two lowest bands were eluted, the salmon-pink band as a yellow solution and the purple band as an orange solution. These two eluates were collected together and the isolation of chrysophanol from them is described on p. 64.

After the washing with benzene the columns were scraped out from below, leaving in place the two upper bands, and fresh $MgCO_3$ was packed into the spaces so made. Washing with methanol (100 ml.) developed the chromatogram into four bands which were from top downwards: (i) a narrow, greyish purple band, (ii) a broad ochre-yellow band, (iii) a pale purple band, and (iv) a bright yellow band. Bands (i), (iii) and (iv) were discarded. The broad ochre-yellow band (ii) was separated mechanically and, while still moist with methanol, was added with stirring to ice-cold, aqueous 2 N-HCl. The brown suspension was extracted twice with large volumes of ether. *Flavoskyrin* (0·45 g.) separated as large yellow needles when the light brown-yellow ethereal solutions were concentrated.

The mycelium, after extraction with benzene in the cold as described above, was re-extracted with the same solvent in a Soxhlet for 5 hr., the solvent being changed at the end of each hour. The five orange-brown solutions so obtained were set aside for a few days and the reddish brown solid that had precipitated was collected as fraction A (4·91 g.) which contained *skyrin* and a colourless substance. The benzene filtrate from fraction A was chromatographed in the same way as is described above for the cold benzene extracts and gave a similar series of bands on the columns. The ochre-yellow bands, treated as described above, yielded flavoskyrin (1·70 g.). Chrysophanol was also recovered from the yellow eluates from these columns (see p. 64).

The benzene-extracted mycelium was now dried and the remainder of the colouring matters present in it were removed by extraction in a Soxhlet with two lots of acetone (5 hr., 7 hr.). The first extract was an intense-brown colour, the second rather lighter. The two solutions were evaporated to 500 and 250 ml. respectively, and the dirty, amorphous material which separated was removed (5·4 g.). The combined brown filtrates, evaporated to dryness *in vacuo*, yielded a very sticky, brown solid as fraction B (24·3 g.), consisting mainly of skyrin.

Purification of crude skyrin

Neither of the two crude fractions of skyrin (fractions A and B) could be purified by direct crystallization but were conveniently purified through the acetate. Thus fraction A (1 part) was added slowly to a mixture of acetic anhydride (10 parts by vol.) and fused sodium acetate (2 parts) at 100°. The greenish brown mixture was maintained at this temperature for 2 hr. with frequent shaking and was then cooled, poured into ice-water with stirring and gave crude *hexaacetylskyrin* as a pale yellow-green solid (1·3 parts). A portion of this (1·0 g.) crystallized from glacial acetic acid (8 ml.) as greenish yellow needles (0·63 g.) which on further purification melted at 293–295°.

Fraction B on acetylation in the same way as fraction A and with the same proportions of reagents gave an almost black solid on pouring into ice-water. This material contained a little fat which was separated from the dried, crude acetate by washing it with light petroleum (b.p. 40–60°). The washed crude acetate (26·2 g.) obtained from 24·3 g. of fraction B crystallized from glacial acetic acid (105 ml.) to

give hexaacetylskyrin as greenish yellow needles (6·7 g.), m.p. 292–294° after further purification. The acetates prepared from fractions A and B were identical in properties and gave no depression in melting point on admixture.

The acetate as prepared above had a slightly greenish yellow colour and still contained a trace of material which was readily soluble in cold aqueous N-NaOH. It was readily purified as follows. A portion (0·50 g.) was dissolved in $CHCl_3$ (50 ml.) and the slightly green-yellow solution was shaken with aqueous N-NaOH (3 × 20 ml.). The dark to light purple extracts were discarded and the now orange-yellow $CHCl_3$ solution was washed with water, filtered and evaporated to 4 ml. Warm ethanol (25 ml.) was added with shaking causing the rapid formation of clusters of fine yellow needles of hexaacetylskyrin (0·40 g.), which appear pure yellow in bulk, m.p. 294–295° with darkening. Recrystallization from $CHCl_3$-ethanol in a similar manner raised the melting point to a constant figure of 295–296°.

Pure hexaacetylskyrin (0·20 g.) was hydrolysed in a stream of N_2 by heating with aqueous N-NaOH (20 ml.) for 1 hr. The purple solution was cooled, acidified with 2N-HCl (15 ml.) and extracted with ether (300 ml.). The dark-orange ethereal solution was washed with water, filtered and evaporated to 10 ml. Skyrin separated as clusters of reddish orange, pointed rods (0·11 g.), melting point above 380°. (Found, on material dried in a high vacuum at 150°: C, 66·7, 66·7; H, 3·5, 3·4; *C*-CH_3, 7·6. $C_{30}H_{18}O_{10}$ requires C, 66·9; H, 3·4; 2*C*-CH_3, 5·6%.)

General properties of skyrin

Skyrin as prepared above slowly sublimes, with decomposition, in a high vacuum at 300–320°. Its ethanolic solution gives an intense brown-red colour with $FeCl_3$, which is almost unchanged on addition of water. It dissolves in cold conc. H_2SO_4 to give an initially deep cherry-red solution. Within a few seconds of solution taking place the colour becomes browner and finally clears to a stable emerald green, the whole change taking about 1 min. Skyrin is slightly soluble in dioxan and acetone and very slightly soluble in ether, ethanol, $CHCl_3$ and glacial acetic acid. It is insoluble in 0·5N-$NaHCO_3$ but dissolves readily in N-NH_3, N-Na_2CO_3 and N-NaOH giving deep-purple solutions which are stable in air. When portions of an ethereal solution of skyrin were shaken with equal volumes of 0·2M borate or phosphate buffers the following changes were observed. The aqueous layer was colourless at pH 8·2 or less, very pale purple at pH 8·5 and strong purple at pH 9·0. At pH 10·0 or above the ether layer was quite colourless. An acetone solution of skyrin, passed through a column of MgO, gives a single reddish purple band which spreads slightly and becomes bluer when washed with methanol.

Skyrin is not readily crystallized from any of the solvents tested and the preparation of pure crystalline material is made more difficult by the fact that skyrin occurs in two crystalline forms which give somewhat different reactions with cold conc. H_2SO_4. It is unusual for either form to separate from a solvent unaccompanied by at least traces of the other form. It has already been noted that amorphous skyrin, obtained by hydrolysis of its acetate, when crystallized from ether gives reddish orange, pointed rods. These crystals, as well as amorphous skyrin, when dissolved in cold conc. H_2SO_4 give a solution which is initially deep cherry red changing in about 1 min. to a stable emerald green. On the other hand, when skyrin is crystallized by evaporating a hot saturated solution in either ethanol (ochre-yellow, pointed rods) or acetone (brownish yellow, elongated hexagonal plates) the resulting crystals in each case give an immediate emerald-green solution in cold conc. H_2SO_4.

The two forms of skyrin are readily interconvertible. Thus skyrin obtained by crystallization from ether and giving the red to green reaction with cold conc. H_2SO_4, when crystallized from acetone was obtained as yellow plates giving the immediate emerald-green H_2SO_4 colour. Similarly, when the crystals obtained from acetone were dissolved in aqueous N-NaOH and the purple solution was immediately acidified and extracted with ether, red-orange rods were obtained on evaporation of the ethereal solution. These crystals dissolved in cold conc. H_2SO_4 to a solution initially red and changing to green in 1 min.

Derivatives of skyrin

Hexaacetylskyrin. The preparation of pure hexaacetylskyrin, m.p. 295–296°, is described on p. 60. (Found, on material dried at 100° in a high vacuum: N, S, halogens and OCH_3, nil; C, 63·4, 63·3; H, 3·9, 3·8; CH_3CO, 31·6, 31·8; mol.wt. (cryoscopic in camphor), 807, 814, 813; optical rotation, nil ($c = 0·635$ in $CHCl_3$). $C_{42}H_{30}O_{16}$ requires C, 63·8; H, 3·8; $6CH_3CO$, 32·7%; mol.wt. 791.) Hexaacetylskyrin crystallizes as pale-yellow, pointed needles which are very readily soluble in $CHCl_3$, readily soluble in acetone, and slightly soluble in dioxan, benzene, ether and glacial acetic acid. It does not dissolve in 0·5N-$NaHCO_3$ or in N-Na_2CO_3 but dissolves slowly in cold aqueous N-NaOH to a purple solution. Its solution in cold conc. H_2SO_4 is deep cherry red at first passing through brown to emerald green in approx. 1 min. Its ethanolic solution gives no colour with $FeCl_3$. It does not sublime in a high vacuum below 220°.

Hydrolysis of acetate and re-acetylation. Dried hexaacetylskyrin (0·9818 g.) was heated on the boiling-water bath for 3 hr. in a stream of N_2 with aqueous NaOH (100 ml., 0·9964N). N-H_2SO_4 (110 ml.) was then added from a burette to the dark-purple solution and the flask was kept in the boiling water for 10 min. to coagulate the orange precipitate. When cool this was collected by filtration, washed and dried (0·653 g.; theor. 0·679 g.). The filtrate and washings were titrated to phenolphthalein with the standard NaOH used for hydrolysis. 17·62 ml. NaOH were required, corresponding to 31·6% CH_3CO· in the substance. The acetic acid in this neutralized solution was determined by a distillation process; net titre, 7·21 ml. N-NaOH ≡ CH_3CO· 31·6%. A portion of the crude orange pigment (0·03 g.) was dissolved in acetic anhydride containing 2% v/v, conc. H_2SO_4 (1 ml.) and heated to 100° for 5 min. The product recovered by pouring into water was crystallized twice from $CHCl_3$ and ethanol (0·5 + 10 ml.), yielding fine yellow needles (0·02 g.), m.p. 294–296° not depressed on admixture with hexaacetylskyrin, m.p. 295–296°.

Skyrin pyridine salt. Skyrin (0·30 g.) dissolved readily in hot pyridine (10 ml.) to a red-brown solution from which, on cooling, bright-red, rhomboidal crystals were deposited (0·31 g.). These were recrystallized from pyridine (10 ml.) to give *skyrin pyridine salt* (0·19 g.). (Found on material dried in a high vacuum at room temperature: C, 68·6; H, 4·5; N, 4·3. $C_{10}H_{28}O_{10}N_2$, i.e. $C_{30}H_{18}O_{10}$, $2C_5H_5N$, requires C, 69·0; H, 4·1; N, 4·0%.) The substance blackens without melting when heated. Its reactions with alkalis are the same as those of the parent pigment and in cold conc. H_2SO_4 it gives a red solution, changing to green.

Dimethylskyrin. Hexaacetylskyrin (0·50 g.) was boiled under reflux for 2 hr. with methanol (100 ml.) and conc. H_2SO_4 (3 ml.). A greenish yellow solid began to separate after 20 min., and at the end of the reaction period the mixture was cooled and the precipitate was collected (0·37 g.). A portion of this (0·17 g.) was crystallized from dioxan (70 ml.) to give *dimethylskyrin* as orange-yellow, rhomboidal plates (0·09 g.) which blacken on heating but do not melt below 360°. (Found on material dried in a high vacuum at 150°: C, 67·3; H, 4·1; OCH_3, 11·1. $C_{32}H_{22}O_{10}$ requires C, 67·8; H, 3·9; $2OCH_3$, 11·0%.) Dimethylskyrin crystallizes unchanged from pyridine but is partially hydrolysed in hot glacial acetic acid. It is readily soluble in $CHCl_3$ but is almost insoluble in methanol, acetone and ethyl acetate. It is insoluble in cold aqueous N-Na_2CO_3, but dissolves readily in aqueous N-NaOH to an orange solution which quickly passes through red and brown to purple. Material recovered from this purple solution dissolves in N-Na_2CO_3 and conc. H_2SO_4 to give solutions identical in colour with those of skyrin. Dimethylskyrin dissolves in cold conc. H_2SO_4 to give a green solution immediately.

Diethylskyrin. Hexaacetylskyrin (1·0 g.) was boiled under reflux for 2 hr. with ethanol (150 ml.) containing conc. H_2SO_4 (4·5 ml.). The solid dissolved to a dark-orange solution from which a light-brown powder precipitated on further heating. This solid was collected (0·73 g.), treated with charcoal in $CHCl_3$ solution and precipitated by the addition of ethanol (5 vol.). The product was crystallized from dioxan (70 ml.) giving *diethylskyrin* (0·49 g.) as yellow rhomboidal plates appearing slightly greenish in bulk. (Found on material dried in a high vacuum at 160–170°: C, 68·3, 68·3; H, 4·4, 4·5; OC_2H_5, 15·2, 15·35. $C_{34}H_{26}O_{10}$ requires C, 68·7; H, 4·4; $2OC_2H_5$, 15·2%.) On heating, diethylskyrin blackens in the neighbourhood of 300°, but does not melt below 360°. It is similar in general properties and colour reactions to the methyl analogue described above. Diethylskyrin was also formed when a solution of skyrin (0·27 g.) in boiling ethanol (300 ml.) was cooled and left in the refrigerator for 2 weeks.

Hexamethylskyrin. A mixture of skyrin (0·37 g.), dry acetone (100 ml.), dimethyl sulphate (3·7 ml.) and anhydrous K_2CO_3 (7·4 g.) was boiled under reflux for 5 hr. The mixture was concentrated and poured into water (1 l.) containing 2N-HCl (100 ml.). The amorphous, orange solid which separated (0·42 g.) was dissolved in $CHCl_3$ and freed from traces of incompletely methylated material by shaking with dilute NaOH. The remaining alkali-insoluble material could not be crystallized but was purified by sublimation in a high vacuum at 300–310° to give *hexamethylskyrin* as a microcrystalline orange powder, m.p. 370–372°. (Found: C, 68·8, 68·9; H, 4·8, 4·85; OCH_3, 28·2; *C*-CH_3, 3·3. $C_{36}H_{30}O_{10}$ requires C, 69·5; H, 4·9; $6OCH_3$, 29·9; 2*C*-CH_3, 4·8%.) Demethylation of the substance in a Zeisel estimation proved to be very difficult. Hexamethylskyrin is sparingly soluble in most solvents but very readily dissolves to an orange solution in $CHCl_3$. It is insoluble in alkalis but in cold conc. H_2SO_4 it dissolves to a stable red-purple solution, indistinguishable in colour from that given by trimethylemodin.

Oxidation of skyrin. Isolation of skyric acid

A solution of hexaacetylskyrin (2·0 g.) in glacial acetic acid (40 ml.) and acetic anhydride (40 ml.) was maintained at 57–58°. A solution of CrO_3 (4·0 g.) in water (3·2 ml.) and glacial acetic acid (40 ml.) was added in small portions during 0·5 hr., in which time the solution became green-brown. After heating for a further 3 hr. at 66–68° the pure green solution was cooled and poured into cold water (1·5 l.) with stirring. The pale green-yellow precipitate (1·5 g.) was separated by filtration after stirring for 2 hr. When the original method of Fischer & Gross (1911) was used, i.e. pouring the reaction mixture into hot water and allowing to stand overnight, the yield from 2·0 g. starting material was 1·24 g.

The crude product was freed from a trace of incompletely oxidized material by shaking a solution of it in $CHCl_3$ (250 ml.) with 0·25N-$NaHCO_3$ (250, 100 ml.). The extracts were quickly acidified, the orange precipitate was collected (1·27 g.) and re-acetylated by heating with acetic anhydride (6 ml.) containing conc. H_2SO_4 (two drops) at 100° for 5 min. Yield 1·38 g.

A portion of the re-acetylated material (0·61 g.) was crystallized three times from methanol (40, 62, 55 ml.), yielding *hexaacetylskyric acid* (0·35 g.) as yellow oblique-ended rods m.p. 231–232° with effervescence and blackening. (Found on material dried in a high vacuum at 100°: C, 59·0; H, 3·1. $C_{42}H_{26}O_{20}$ requires C, 59·3; H, 3·1%.) Crystallization from glacial acetic acid was found to cause slight hydrolysis, leading to an orange-yellow product, m.p. 194–200°. Hexaacetylskyric acid dissolves in conc. H_2SO_4 to an initially deep cherry-red solution which changes during about 4 min. through brown to green. It dissolves slowly in 0·5N-$NaHCO_3$ to an orange solution changing to red in a few hours. It dissolves readily in N-Na_2CO_3, the colour changing from orange to red in about 5 sec. and then to purple during several hours. The solution of the substance in N-NaOH is initially red and changes to purple in 1 min.

Hexaacetylskyric acid (0·73 g.) was hydrolysed under N_2 with aqueous N-NaOH (100 ml.) on the boiling-water bath for 1 hr. 2N-HCl (75 ml.) was added to the purple solution and the mixture was heated again to coagulate the precipitate, which changed from orange to ochre-yellow during the heating. When separated, the crude skyric acid was obtained as an amorphous, ochre-yellow powder (0·57 g.) which did not melt below 370°. It was almost insoluble in nearly all organic solvents and could not be crystallized. It dissolved in 0·5N-$NaHCO_3$ to a purple-red solution and in N-Na_2CO_3 and N-NaOH to a purple solution. It gave a green solution at once when dissolved in cold conc. H_2SO_4.

Skyric acid was obtained crystalline as follows. Hexaacetylskyric acid (0·05 g.) was hydrolysed as described above and the cooled alkaline solution was acidified and extracted with ether (250 ml.). Bright orange-red rods and lozenge-shaped crystals (0·03 g.) which do not melt below 370° were obtained by evaporation of the ether extract (cf. the preparation of crystalline skyrin, p. 61). (Found on material dried in a high vaccum at 130°: C, 55·0; H, 3·7. $C_{30}H_{20}O_{17}$, i.e. $C_{30}H_{14}O_{14}$, $3H_2O$, requires C, 55·2; H, 3·1%.) This material gave colour reactions with alkalis which were identical with those given by the amorphous material prepared as described above, but in cold conc. H_2SO_4 it dissolved to a red solution which changed through brown to green in about 4 min.

Dimethyl skyrate. Hexaacetylskyric acid (0·50 g.) was boiled under reflux with methanol (25 ml.) containing 3%, v/v, H_2SO_4. The substance dissolved and the yellow solution quickly turned orange. An orange crust, probably skyric acid, formed on the walls of the flask during the first hour. This slowly dissolved and the mixture became a lighter

colour as boiling continued. After boiling for 5 hr. the mixture was cooled and the amorphous yellow solid which separated was collected (0·30 g.). A portion of this (0·20 g.) was boiled with dioxan (20 ml.) and the cloudy solution clarified by filtration through kieselguhr. Addition of methanol (20 ml.) to the clear filtrate yielded *dimethyl skyrate* as yellow oblique-ended rods (0·09 g.) which blacken on heating above 200° but do not melt below 370°. (Found on material dried to constant weight in a high vacuum at 120°: C, 61·0; H, 3·4; OCH_3, 10·4. $C_{32}H_{18}O_{14}$ requires C, 61·3; H, 2·9; 2 OCH_3, 9·9%.) Dimethyl skyrate is insoluble in 0·5N-$NaHCO_3$ but dissolves in N-Na_2CO_3 or N-NaOH to orange solutions that quickly change through red to purple. It gives an immediate green colour when dissolved in cold conc. H_2SO_4.

Reductive cleavage of skyrin. Isolation of Frangula *emodin*

Vacuum-dried amorphous skyrin (0·5918 g.), prepared by alkaline hydrolysis of hexaacetylskyrin, was dissolved in aqueous N-Na_2CO_3 (100 ml., accurately measured), and a solution of sodium dithionite, $Na_2S_2O_4$ (10 g.), in water (50 ml.) was added through a filter. After gentle shaking for 5 min. a volume of N-HCl equivalent to the Na_2CO_3 present was cautiously added. The orange solid thus precipitated was collected on a pad of kieselguhr, washed with water and dilute HCl, and dried. The product was separated by extraction of the kieselguhr in a Soxhlet, first with ether (30 hr.) and then with methanol (8 hr.). The dried ether extract (0·497 g.) was dissolved in the methanol extract and three crops of crystals (total weight 0·573 g.) were obtained by evaporation. Removal of the solvent from the final mother liquor left a sticky, brown solid (0·093 g.).

The crystalline material and the dried mother liquor were sublimed in a high vacuum at 175–185° giving orange sublimates, total weight 0·5357 g., equivalent to 90·5% of the starting material. The loss in weight on sublimation is approximately equivalent to 1 mol. methanol/1 mol. emodin. The sublimate was crystallized from $CHCl_3$ (300 ml.) and toluene and was resublimed in a high vacuum giving a product, m.p. 255–256°, not depressed on admixture with authentic *Frangula* emodin, m.p. 256°. (Found on sublimed material: C, 66·4; H, 3·7. Calc. for $C_{15}H_{10}O_5$: C, 66·7; H, 3·7%.)

A portion of the sublimate (0·26 g.) was methylated by boiling in acetone (60 ml.) with dimethyl sulphate (2·6 ml.) and anhydrous K_2CO_3 (5·2 g.). The methylated product was crystallized from ethanol (40 ml.) yielding glistening yellow needles (0·19 g.), m.p. 225°, either alone or mixed with authentic trimethylemodin, m.p. 225°. (Found on material dried in a high vacuum at 90°: C, 69·1, 69·3; H, 5·1, 5·3; OCH_3, 29·8, 29·7. Calc. for $C_{18}H_{16}O_5$: C, 69·2; H, 5·2; 3OCH_3, 29·8%.)

When a solution of emodin in N-Na_2CO_3 was treated with $Na_2S_2O_4$ the colour of the solution changed from red to orange and emodin could be isolated unchanged from this solution by extraction with $CHCl_3$.

Reductive cleavage of skyric acid. Isolation of emodic acid

Vacuum-dried amorphous skyric acid (0·399 g.) was dissolved in N-Na_2CO_3 (100 ml.) by warming. To the cooled solution was added a filtered solution of $Na_2S_2O_4$ (7 g.) in water (70 ml.). The colour changed from purple to dark orange. After 10 min. N-HCl (100 ml.) was cautiously added and the resulting brown-orange precipitate was collected on a pad of kieselguhr. When dry the product was extracted from the kieselguhr by boiling repeatedly with glacial acetic acid. The acetic acid solution was evaporated to dryness and sublimed in a high vacuum. The sublimate was shaken with $CHCl_3$ (150 ml.) and 0·25N-$NaHCO_3$ (350 ml.). The $NaHCO_3$-soluble fraction was recovered by acidification (0·272 g. ≡ 68% of starting material). It crystallized from glacial acetic acid as long, dark-orange needles, m.p. 363–365° alone or mixed with authentic emodic acid, m.p. 363–365°. When dried in a high vacuum at 200°, the crystals lost 1 mol. acetic acid. (Found, loss on drying = 17·2%. Calc. for $C_{15}H_8O_7$, $C_2H_4O_2$ = 16·7%. Found on dried material: C, 60·5; H, 2·8. Calc. for $C_{15}H_8O_7$: C, 60·0; H, 2·7%.)

To confirm the identity of the product it was converted successively into its methyl ester and its methyl ester triacetate, following the procedure of Anslow *et al.* (1940). The methyl ester was thus obtained as fine, orange needles, m.p. 266–267° alone or mixed with authentic methyl emodate, m.p. 267–268°. The methyl ester triacetate was prepared as yellow needles, m.p. 188–189° alone or mixed with authentic methyl triacetylemodate of the same melting point. (Found on material dried in a high vacuum at 70°: C, 59·3; H, 3·7; OCH_3, 6·85. Calc. for $C_{22}H_{16}O_{10}$: C, 60·0; H, 3·7; 1 OCH_3, 7·05%.)

Flavoskyrin

The separation of flavoskyrin from mycelial extracts as orange- or green-yellow needles, m.p. about 200° (decomp.), has already been described (see p. 60). This crude material (0·20 g.) was purified by dissolving in warm dioxan (25 ml.). The solution was filtered, glacial acetic acid (25 ml.) was added and the dark-orange solution was concentrated *in vacuo* to 15 ml. Orange-yellow needles (0·13 g.) separated and were recrystallized by the same method giving *flavoskyrin* (0·10 g.) as yellow needles, m.p. 214–215° (decomp.). A portion of this material was further purified for analysis by crystallization from acetone plus water giving yellow needles, m.p. 215° (decomp.). (Found on material dried to constant weight in a high vacuum at 150°: C, 66·1, 66·2; H, 4·45, 4·5. $C_{15}H_{12}O_5$ requires C, 66·2; H, 4·4%.)

Flavoskyrin crystallizes as yellow needles, m.p. 215°, with vigorous effervescence, the residue setting to a dark-orange, partly crystalline solid. Flavoskyrin is fairly readily soluble in dioxan, less readily soluble in acetone, $CHCl_3$, ethanol, methanol or glacial acetic acid, and only slightly soluble in benzene and ether. Its solutions in organic solvents are bright yellow in colour.

Flavoskyrin is very slightly soluble in 0·5N-$NaHCO_3$ but dissolves readily in N-Na_2CO_3 and N-NaOH to yellow solutions which turn slightly more orange on standing. Portions of an ethereal solution were shaken with equal volumes of 0·2M borate or phosphate buffers. At pH 6·0 and below the aqueous layer was colourless; at pH 7·2 it was pale yellow and at pH 7·4 the colours of the aqueous and ether layers were of equal intensity; at pH 8·2 the ether layer was pale yellow and at pH 8·5 and above the flavoskyrin was wholly in the aqueous layer. Chromatography in acetone solution on $MgCO_3$ gives a single, uniform, bright-yellow band, the colour of which is stable over long periods, and which shows no dissociation into two bands on prolonged washing.

Flavoskyrin dissolves in cold conc. H_2SO_4 to an initially bright-orange solution which changes during 2 hr. to purple-red. Addition of $FeCl_3$ to an ethanolic solution of flavoskyrin produces an olive-brown colour. Flavoskyrin appears to be unaffected by $Na_2S_2O_4$ either in the solid state or dissolved in $CHCl_3$ or in aqueous Na_2CO_3. No change in colour takes place when Zn dust is added to its solution in glacial acetic acid.

The molecular weight of flavoskyrin. In attempting to determine the molecular weight of flavoskyrin, the usual cryoscopic methods (camphor, dioxan) were found to be inapplicable, because of the thermal instability or the low solubility of the substance. An approximate value was obtained by the isothermal distillation method of Barger (1904), using azobenzene as standard and dioxan as solvent. Here again, however, the low solubility was a handicap, the molar concentrations attainable being only about one-quarter of the lowest used by Barger. Many observations suggest a value of about 350 for the molecular weight of flavoskyrin. (Theory for $C_{15}H_{12}O_5$, 272; for $C_{30}H_{24}O_{10}$, 544.)

Acetylation of flavoskyrin. Flavoskyrin was acetylated with pyridine and acetic anhydride (2:1, v/v) at room temperature for 3 days. The creamy orange acetylation product, m.p. 205–215°, could not be crystallized. It was insoluble in N-NaOH except on standing and did not sublime or appear to decompose when heated to 230° in a high vacuum.

Methylation of flavoskyrin. Treatment of flavoskyrin with dimethyl sulphate and K_2CO_3 in boiling acetone for 7 hr. yielded a yellow powder, m.p. 125–136°, which could not be purified. It was insoluble in N-NaOH and gave a stable red solution in cold conc. H_2SO_4.

Decomposition of flavoskyrin. Isolation of chrysophanol

(*a*) *Thermal decomposition.* Dried flavoskyrin (47·9 mg.) was heated in a wide test tube in a stream of N_2, freed from O_2 and CO_2, and the issuing gas was led through 0·2N-$Ba(OH)_2$. Wet filter paper was wrapped round the upper parts of the tube while the contents of the tube were heated in an oil bath, first at 205° for 45 min. and then at 212° for 15 min. No CO_2 was evolved, but an orange sublimate had collected on the cooler upper part of the tube. The tube was now connected to a high vacuum pump and the decomposed residue was heated at 130°. By this means the decomposition product remaining in the residue was sublimed out alongside the sublimate formed during the reaction. (The equation $C_{15}H_{12}O_5 \rightarrow C_{15}H_{10}O_4 + H_2O$ requires $C_{15}H_{10}O_4$, 93·4; H_2O, 6·6%. Found: total weight of sublimate, 41·8 mg. = 87·3% of starting material; loss in weight during decomposition (H_2O), 3·2 mg. = 6·7%. The yield of sublimate is therefore 93·5% of the theoretical.) The sublimate was freed from a trace of Na_2CO_3-soluble material and was then crystallized from ethanol (13 ml.) to give golden leaflets (0·04 g.), m.p. 193–194°, raised, by further crystallization from the same solvent, to 194–195° alone or mixed with authentic chrysophanol of the same melting point. (Found on substance dried in a high vacuum at 80°: C, 71·2; H, 4·0. Calc. for $C_{15}H_{10}O_4$: C, 70·9; H, 4·0%.)

(*b*) *Decomposition by conc.* H_2SO_4. Dried flavoskyrin (101·1 mg.) was dissolved in cold conc. H_2SO_4 (5 ml.) and the dark red-orange solution was held at room temperature for 4 hr. with occasional shaking. The colour changed during the first 2 hr. to an intense red-purple. At the end of the reaction period the solution was rinsed into ice-water (150 ml.) and the bright orange-yellow precipitate was extracted into ether (3 × 200 ml.). The ethereal solution was shaken with 0·5N-Na_2CO_3 (2 × 50 ml.) to remove a little by-product, and the material remaining in the ether was sublimed in a high vacuum at 130°. (Yield of sublimate, m.p. 191–194°, 65·8 mg. ≡ 69% of theoretical.) Crystallization of the sublimate from ethanol (21, 20 ml.) yielded chrysophanol as golden leaflets (44 mg.), m.p. 194–195° alone or mixed with authentic chrysophanol of the same melting point. (Found on material dried in a high vacuum at 70°: C, 71·1; H, 3·9. Calc. for $C_{15}H_{10}O_4$: C, 70·9; H, 4·0%.) The ether-extracted dilute H_2SO_4 reaction mixture, which was pale yellow in colour becoming purple on making alkaline, obviously contained water-soluble material which probably consists of anthraquinonesulphonic acids formed as by-products during the decomposition.

Chrysophanol. Isolation from mould mycelium

(*a*) The chromatography of the benzene extracts of the dried mycelium of *P. islandicum*, N.R.R.L. 1175, both that obtained by extraction in the cold and that from hot extraction in a Soxhlet, has already been described (see p. 60). The coloured eluates obtained in each case contained chrysophanol which was isolated in the following manner. The eluate was shaken very gently with dilute aqueous NaOH and the extracts were quickly acidified. The regenerated pigments, now free from fat, were rechromatographed in benzene on $MgCO_3$, giving a clean homogeneous lower band, salmon-pink in colour, from which almost pure chrysophanol was obtained by acidification.

The material from the cold benzene extracts (0·36 g.) was crystallized from ethanol (120 ml.) and then from benzene (10 ml.) as orange leaflets, m.p. 194–195° alone or mixed with authentic chrysophanol of the same melting point. (Found on material sublimed in a high vacuum at 130°: C, 70·7, H, 3·9. Calc. for $C_{15}H_{10}O_4$: C, 70·9; H, 4·0%.)

The material from the hot Soxhlet benzene extracts (0·56 g.) was crystallized from benzene (14 ml.) and sublimed in a high vacuum at 130°. M.p. and mixed m.p. 194–195°. (Found: C, 71·3; H, 3·9. Calc. for $C_{15}H_{10}O_4$: C, 70·9; H, 4·0%.)

(*b*) *P. islandicum*, strain N.R.R.L. 1175, was grown for 24 days on five 1 l. conical flasks each containing 350 ml. of Czapek-Dox solution. The mycelia were then separated from the culture fluid, washed with water and disintegrated in a Top Drive Macerater (Townson and Mercer, Croydon) for 10 min. with benzene (500 ml.). The benzene was separated by filtration and the pulp was extracted twice more with benzene in the macerater. The three dark-orange extracts were combined, washed, dried over Na_2SO_4 and passed through a column of heavy $MgCO_3$ (3·5 × 38 cm.). After washing with benzene the salmon-pink band was separated mechanically, dried in air, acidified with 2N-HCl, and the suspension was extracted with ether. Chrysophanol was separated from accompanying fat by shaking the ethereal solution with 0·5N-NaOH (2 × 50 ml.) and was then regenerated by acidification of the alkaline extract. The orange solid so obtained was sublimed in a high vacuum at 130° and the sublimate was crystallized from light petroleum, b.p. 80–100° (10 ml.), yielding orange leaflets (10 mg.), m.p. 192–194°, not depressed on admixture with authentic chrysophanol, m.p. 194–195°.

SUMMARY

1. Two hitherto undescribed colouring matters, skyrin and flavoskyrin, have been isolated from the mycelium of laboratory cultures of a morphologically typical but biochemically atypical strain of *Penicillium islandicum* Sopp.

2. Skyrin, $C_{30}H_{18}O_{10}$, crystallizes as clusters of orange-red rods or as yellow, hexagonal plates and does not melt below 380°. Several of its derivatives are described.

3. When an alkaline solution of skyrin is treated with sodium dithionite, $Na_2S_2O_4$, it undergoes reductive cleavage into two molecules of emodin, $C_{15}H_{10}O_5$, 4:5:7-trihydroxy-2-methylanthraquinone.

4. Flavoskyrin, $C_{15}H_{12}O_5$, crystallizes as yellow needles, m.p. 215° (decomp.).

5. Flavoskyrin loses a molecule of water to yield chrysophanol, $C_{15}H_{10}O_4$, 4:5-dihydroxy-2-methylanthraquinone, either by heating at its melting point or by the action of cold concentrated sulphuric acid.

6. The chrysophanol already reported as a metabolic product of this strain of *P. islandicum* has been shown not to be an artifact produced, during its isolation, by decomposition of flavoskyrin.

7. Possible structures for skyrin are discussed.

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Chemical Action of X-Rays on Nucleic Acids and Related Substances in Aqueous Systems

2. THE MECHANISM OF THE ACTION OF X-RAYS ON NUCLEIC ACIDS IN AQUEOUS SYSTEMS

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The nature of the chemical changes which occur on irradiation of nucleic acids in aqueous systems with X-rays have been described in Part 1 of this series (Scholes & Weiss, 1953*a*). Evidence was presented to show that these changes were the result of chemical reactions involving the free radicals produced by the absorption of the radiation by the water which is split according to the net process (Weiss, 1944, 1947*a*, *b*):

$$H_2O \text{—}\sim\sim\rightarrow H + OH \qquad (1)$$

In these experiments, the detection and isolation of the products of irradiation were facilitated by employing fairly high doses of X-rays (approx. 1×10^6r).

Previous investigations of the action of X-rays on deoxyribonucleic acid (DNA) solutions, using doses in the region 20 000–100 000r have been essentially concerned with the physical effects of the radiation, for example the decrease in viscosity and of streaming birefringence (cf. Taylor, Greenstein & Hollaender, 1948). No chemical changes have been reported. It was therefore of interest, particularly from a biological standpoint, to investigate the nature of any chemical effects which may result by the use of similarly low, or even lower, doses of radiation. In fact, it was found that ammonia and inorganic phosphate were both formed when solutions of DNA and RNA (ribonucleic acid) were irradiated with doses of the order of 30000r (Scholes & Weiss, 1950). Furthermore, quantitative determination of these products formed under different experimental conditions (e.g. in the presence of oxygen, of hydrogen and *in vacuo*) has yielded information about the nature of the radicals involved in the various processes. In the following, our detailed results are presented and the mechanism of the degradation induced by X-rays of the polynucleotides is discussed.

EXPERIMENTAL

Materials. The commercial samples of (yeast) RNA (BY3) and (thymus) DNA (BT2) were the sodium salts supplied by British Drug Houses Ltd. The sample RNA (BYC) was a purified commercial specimen (cf. Scholes & Weiss, 1953*a*) and contained: N, 16·2; P, 9·2%. RNA (BY3) contained: N, 15·8; P, 9·1%. A specimen of highly polymerized (thymus) DNA (mol.wt. approx. 1×10^6) was kindly supplied by Prof. R. Signer (Berne). A pure but less polymerized sample of DNA, originally prepared by Hammarsten, has also been irradiated.

Adenine, adenylic acid (yeast) and cytidylic acid (yeast) were pure commercial products (Roche Products Ltd.); the pyrimidine nucleotide was further purified by recrystallization from 50% ethanol and had a ratio C/N of 2·98. (Microanalysis by Weiler and Strauss, Oxford.)

Irradiation

The arrangements for irradiation were similar to those described previously (Scholes & Weiss, 1953*a*).

The solutions (100 ml.) were prepared in triply distilled water, ordinary distilled water having been redistilled in an all-glass still from alkaline permanganate and then from phosphoric acid. When required, the solutions were buffered by the following systems: for pH <4, H_2SO_4; pH 4–8, boric acid-borax; pH 5–8, Na_2HPO_4-KH_2PO_4 (Sørensen); pH 8–10, boric acid-NaOH; pH 9–11, Na_2CO_3-borax (Parnas); and for pH >11, NaOH. All reagents were of A.R. grade and minimum quantities of the standard buffers were used (usually about 10 ml. buffer solution/100 ml. solution to be irradiated). Besides irradiations in the presence of air, experiments were also carried out in an atmosphere of O_2 (the solutions being saturated with O_2 by bubbling the gas through the solution for about 30 min.), *in vacuo* and in the presence of H_2 (1 atm.).

Evacuation was effected by passing CO_2 through the solution for a short while and pumping out the vessel by means of a Hyvac oil pump; this process was generally repeated once or twice. In certain cases (e.g. when evacuating alkaline solutions) purified O_2-free N_2 was used in place of CO_2. Commercial 'O_2-free' N_2 was purified for this purpose by passage through Fieser's solution (Fieser, 1924), through a $CuSO_4$ solution and then through a $CaCl_2$ and soda-lime scrubber and finally through a tube containing Cu gauze at approx. 380°. The gas so purified was found to contain less than one part per million of O_2 when tested with a trypaflavine-silica gel (Kautsky & Hirsch, 1935; Franck & Pringsheim, 1943). After the evacuation procedure less than 0·01 ml. of non-condensable gas remained.

For the experiments in the presence of H_2, the vessel containing the solution to be irradiated was first evacuated as described above, and then purified, O_2-free H_2 was slowly admitted to atmospheric pressure. In order to ensure saturation, the gas was slowly bubbled through the solution for a further 30 min.

Quantitative methods

Determination of ammonia. Samples (50 ml.) were analysed according to the method described by Scholes & Weiss (1953*a*).

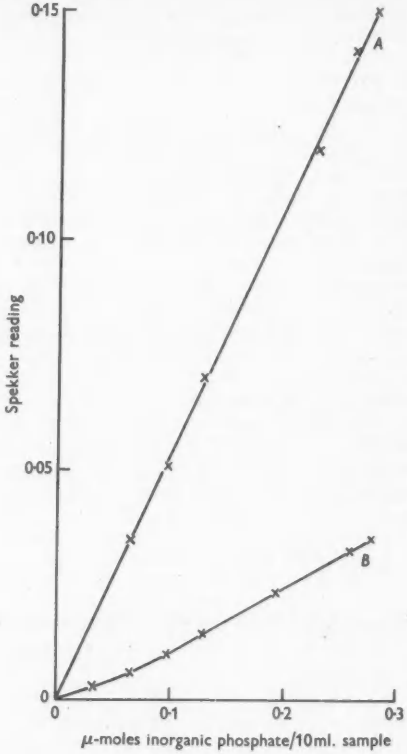

Fig. 1. Calibration curves for the determination of inorganic phosphate by the methods of Berenblum & Chain (1938) (curve *A*) and Lowry & Lopez (1946) (curve *B*). Samples (10 ml.) treated with reagents to a final volume of 25 ml. Optical densities measured with a Spekker photoelectric absorptiometer (Ilford 608 filter). Calibration constructed with KH_2PO_4.

Determination of inorganic phosphate. Of the various available methods for the determination of inorganic phosphate in the presence of 'labile' phosphate, those of Lowry & Lopez (1946) and Berenblum & Chain (1938) were used. Both these methods were found to be suitable for the determination of small amounts of inorganic phosphate; Fig. 1 shows the corresponding calibration curves. In all cases 10 ml. samples were analysed, measurements being made in triplicate and compared against the corresponding blanks.

With the Lowry & Lopez procedure, the irradiated nucleate solutions (which were analysed without prior removal of unchanged nucleic acid) showed some lag in colour development; but the maximum was attained after about 45 min. The method was found to be reproducible (to approx. $\pm 5 \times 10^{-9}$ mole P/10 ml. sample) if, in all cases, the extinction was measured always at the same time (45 min.) after the addition of the reagents.

The Berenblum & Chain method was found to have certain advantages; interfering substances which hindered colour development were removed, the extinction for a given amount of P was much greater than that given by the Lowry & Lopez procedure and the blank values were very low. Measurements were reproducible to approx. $\pm 2 \times 10^{-9}$ moles P/10 ml. sample.

RESULTS

Formation of ammonia

The yields of ammonia from 0·05 % (w/v) solutions of RNA (BY 3), DNA (Signer A) and adenine, produced on irradiation with different doses and under different specified conditions, are shown in Figs. 2–4, respectively. The yields were proportional to the dose (with a 'break' in the presence of air) and in all three cases there was the same sequence in the relative positions of plots of yield versus dose, that is, the yields decreased on passing from irradiations in the presence of oxygen to those *in vacuo*, the lowest values being obtained in the presence of molecular hydrogen. This particular sequence indicates that the liberation of ammonia from the nucleic acids is the result of an attack by oxidative radicals on the constituent bases. This follows from a consideration of the nature of the radicals present under these various experimental conditions: in evacuated solutions, there are the H atoms and the OH radicals, produced according to process (1); in the presence of molecular oxygen the H atoms react as follows,

$$H + O_2 \rightarrow HO_2, \qquad (2)$$

thus being transformed, to a greater or lesser extent, into HO_2 radicals; in the presence of molecular hydrogen the OH radicals can react as follows,

$$H_2 + OH \rightarrow H_2O + H, \qquad (3)$$

thus giving H atoms to a greater or lesser extent. A process involving oxidative radicals will therefore be enhanced in the presence of oxygen and inhibited in the presence of hydrogen. The observed 'breaks' in the graphs of ammonia yield against dose in the presence of air (cf. Figs. 2–4) represent the points at which practically all the atmospheric oxygen in the solution has been used up.

According to Gulland (1947), the highly viscous character of DNA solutions may be due, in part, to the presence of hydrogen bonding between amino and hydroxyl groups. The attack by the oxidative radicals on the component bases, with subsequent elimination of amino groups, should destroy this particular type of hydrogen bonding; this could account for part of the decrease in structural viscosity and of streaming birefringence observed on irradiation of these solutions.

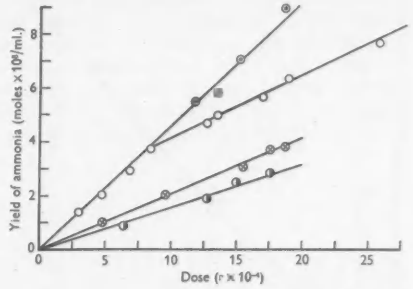

Fig. 2. Formation of ammonia by irradiation of aqueous solutions of RNA (BY 3) with 200 kv X-rays. RNA soln., 0·05 %, w/v; pH ~ 5. ⊙, in oxygen (1 atm.); ○, in air; ⊗, *in vacuo*; ◑, in hydrogen (1 atm.).

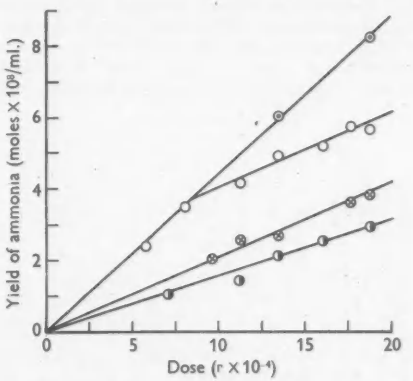

Fig. 3. Formation of ammonia by irradiation of aqueous solutions of DNA (Signer A) with 200 kv X-rays. DNA soln., 0·05 %, w/v; pH ~ 6·5. ⊙, in oxygen (1 atm.); ○, in air; ⊗, *in vacuo*; ◑, in hydrogen (1 atm.).

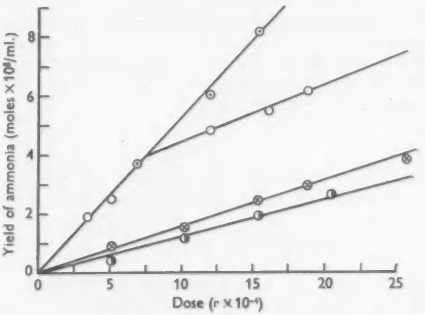

Fig. 4. Formation of ammonia by irradiation of aqueous solutions of adenine with 200 kv X-rays. Adenine soln., 0·05 %, w/v; pH = 7·0 (phosphate buffer). ⊙, in oxygen (1 atm.); ○, in air; ⊗, *in vacuo*; ◑, in hydrogen (1 atm.).

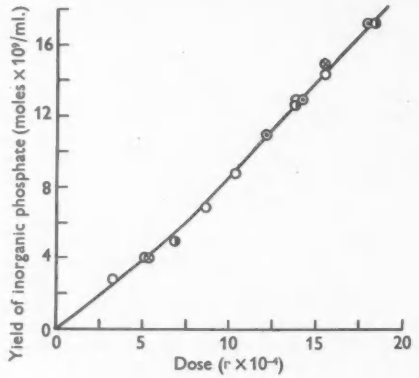

Fig. 5. Formation of inorganic phosphate by irradiation of aqueous solutions of RNA (BY3) with 200 kv X-rays. RNA soln., 0·05%, w/v; pH ~ 5. Phosphate determined by method of Lowry & Lopez (1946). ⊙, in oxygen (1 atm.); ○, in air; ⊗, *in vacuo*; ◑, in hydrogen (1 atm.).

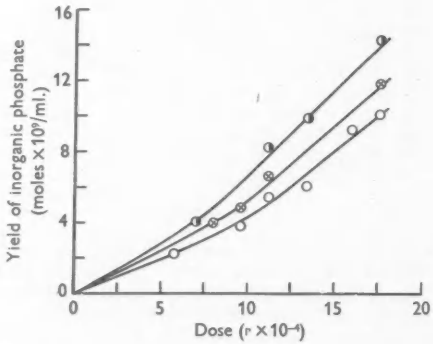

Fig. 6. Formation of inorganic phosphate by irradiation of aqueous solutions of DNA (Signer A) with 200 kv X-rays. DNA soln., 0·05%, w/v; pH ~ 6·5. Phosphate determined by method of Lowry & Lopez (1946). ○, in air; ⊗, *in vacuo*; ◑, in hydrogen (1 atm.).

Table 1. *Formation of inorganic phosphate by irradiation of* DNA (*Hammarsten*) *with* 200 kv *X-rays*

(DNA soln., 0·05%, w/v.)

Dose ($r \times 10^{-5}$)	Yield of inorganic phosphate (moles × 10^8/100 ml.) In air	*In vacuo*	In hydrogen (1 atm.)
1·28	0·855	0·985	1·57
1·76	1·39	1·48	1·96

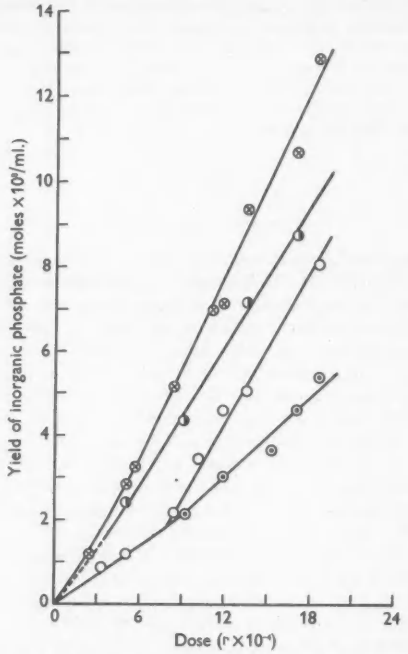

Fig. 7. Formation of inorganic phosphate by irradiation of aqueous solutions of yeast adenylic acid with 200 kv X-rays. Adenylic acid soln., 0·05%, w/v; pH = 3·3. Phosphate determined by method of Lowry & Lopez (1946). ⊙, in oxygen (1 atm.); ○, in air; ⊗, *in vacuo*: ◑, in hydrogen (1 atm.).

Formation of inorganic phosphate

The yield/dose curves for the formation of inorganic phosphate from RNA (BY3) and from DNA (Signer A) solutions are shown in Figs. 5 and 6. There was no observable difference in the yields of inorganic phosphate when RNA solutions were irradiated under the different conditions indicated in Fig. 5. In the case of DNA, on the other hand, phosphate production was enhanced when atmospheric oxygen was excluded from the solution and, in fact, the highest yields were obtained in the presence of molecular hydrogen. Irradiation of a DNA sample prepared by Hammarsten gave results (Table 1) which were similar to those obtained with the Signer acid. Thus, on the basis of the principles outlined above, it appears that the hydrogen atoms may play some part in the degradation of the nucleic acids. If this is the case, it would appear that, with RNA solutions, hydrogen atoms and oxidative radicals are equally effective dephosphorylating agents, whilst with DNA, hydrogen atoms appear to be more reactive.

Fig. 7 records the results obtained from a study of the dephosphorylation induced by X-radiation of yeast adenylic acid in aqueous solution. The yields of inorganic phosphate were, in general, higher than those obtained from the nucleic acids, but, the break in the 'air-curve' and the greater yields *in vacuo* again suggest some role of the hydrogen atoms in the degradation process. The position of the 'hydrogen' curve, that is midway between curves referring to air and vacuum conditions, is a somewhat unusual feature: this may suggest that while some direct dephosphorylation by the radicals can take place, initial attacks by oxidative radicals may enhance subsequent reductive phosphate release.

The curves of phosphate yield against dose in general seem to approach linearity only after a certain time of irradiation and this may indicate a building up of some more reactive species. However, inorganic phosphate is already produced with relatively very low doses, and, in fact, could be detected in RNA solutions even after a dose of 4000r and in yeast adenylic acid solutions after only 2000r.

As the figures reveal, the yields of inorganic phosphate from the nucleic acids are comparatively small but this is not unexpected, as multiple attacks on pentose residues may be required to liberate the bound phosphate groups. From less highly polymerized nucleic acids, where there are relatively more terminal phosphate groups, one may expect greater yields of inorganic phosphate. This, for example, is demonstrated in Fig. 8, which shows a correlation between the yields of phosphate and the state of polymerization of different DNA preparations; the lowest yields were obtained from the highly polymerized Signer acid and the highest from the degraded commercial sample. These observations are in keeping with our general concepts discussed below.

In the case of nucleate solutions, the yields of inorganic phosphate measured by the method of Berenblum & Chain (1938) were always lower than those obtained by the Lowry & Lopez (1946) procedure. Fig. 9, for example, shows the yields of inorganic phosphate obtained by these two different methods when applied to some irradiated RNA solutions. Such evidence lends support to the view that on irradiation of aqueous solutions of the nucleic acids there is a primary formation of some labile phosphate esters, these being subsequently decomposed to some extent under the conditions prevailing in the Lowry & Lopez method. Recent experiments by Weil-Malherbe & Green (1951) have indeed shown that such labile esters may easily decompose in the presence of molybdate as used in this determination, whereas, under the conditions of Berenblum & Chain this molybdate catalysis may not play an important part. Additional experimental evidence for the presence of labile phosphate esters arises from our recent observations that considerable amounts of inorganic phosphate can be liberated on subsequent (acid) hydrolysis of the irradiated RNA and DNA solutions (Scholes & Weiss, 1953*b*). For example, it has been found that after doses of the order of 9×10^4r (in the presence

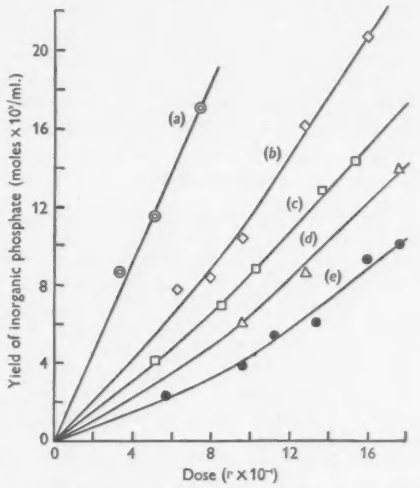

Fig. 8. Formation of inorganic phosphate by irradiation of aqueous solutions of different nucleic acids and of yeast adenylic acid with 200 kv X-rays in the presence of air. Soln., 0·05%, w/v. Phosphate determined by method of Lowry & Lopez (1946). ((*a*) pH = 3·3; (*c*) pH ~ 5; (*b*), (*d*) and (*e*) pH ~ 6·5.) (*a*) yeast adenylic acid; (*b*) DNA (BT2); (*c*) PNA (BY3); (*d*) DNA (Hammarsten); (*e*) DNA (Signer A).

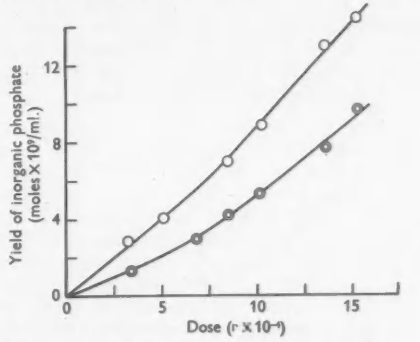

Fig. 9. Formation of inorganic phosphate by irradiation of aqueous solutions of RNA (BY3) with 200 kv X-rays in the presence of air. RNA soln., 0·05%, w/v; pH ~ 5. Phosphate determined by methods of Lowry & Lopez (1946) (○) and Berenblum & Chain (1938) (◎) respectively.

of oxygen) the additional amounts of inorganic phosphate liberated on hydrolysis are about 15 times the amount of free phosphate formed during the irradiation. These observations may be of considerable significance in connexion with the so-called 'after-effect' (the decrease in viscosity of DNA solutions which occurs after cessation of irradiation) and suggest that this effect may be due, at least in part, to slow hydrolytic fission of labile phosphate ester bonds, thus leading to further depolymerization. This is also in agreement with the fact (Taylor *et al.* 1948) that the 'after-effect' is strongly temperature-dependent, as would be expected in the case of a relatively slow hydrolytic process.

Effects of pH

It has already been reported that the amount of ammonia produced from the purine bases depends upon pH (Scholes & Weiss, 1953*a*). Fig. 10 shows the pH-dependence in adenine solutions (0·05%, w/v) which have received a (constant) dose of 102900r (*in vacuo*). While there was little change in the ammonia yields at higher pH's, a fairly marked increase was observed in the neighbourhood of pH 4, tending to reach a maximum in more acid solution. A similar behaviour was exhibited by adenosine solutions.

In the case of the ribonucleotides and the nucleic acids, on the other hand, the pH-dependence was more complex. Figs. 11–13 show the results obtained from irradiation of yeast adenylic acid, yeast cytidylic acid and RNA (BYC) solutions respectively. Although the yields differ somewhat (the highest was obtained from the pyrimidine nucleotide) the similarity in the shape of these curves is quite marked, each exhibiting minima and maxima in the regions of pH 4–5 and pH 8–9 respectively. Thus, the presence of the phosphoryl group exerts a characteristic influence on the pH-dependence of the deamination, this being particularly pronounced in the neutral range.

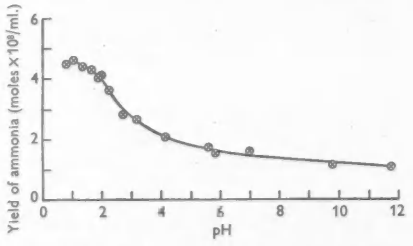

Fig. 10. pH dependence of the formation of ammonia from solutions of adenine irradiated with 200 kv X-rays *in vacuo*. Adenine soln., 0·05%, w/v; total dose, $1{\cdot}03 \times 10^5$r units.

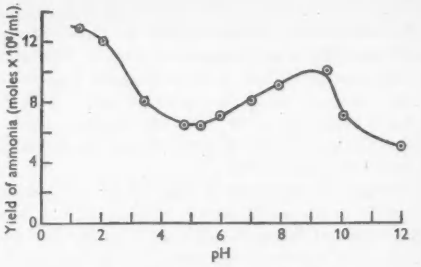

Fig. 11. pH dependence of the formation of ammonia from aqueous solutions of yeast adenylic acid irradiated with 200 kv X-rays in the presence of oxygen (1 atm.). Yeast adenylic acid soln., 0·05%, w/v; total dose, $9{\cdot}45 \times 10^4$r units.

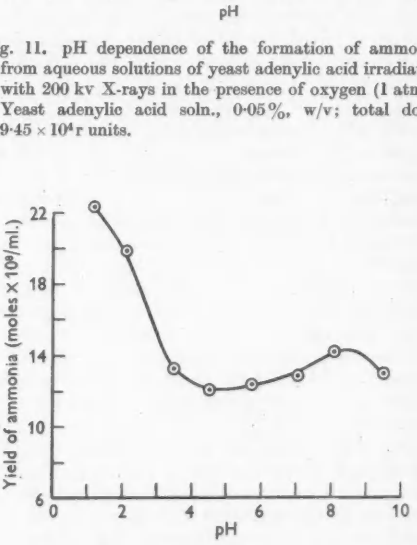

Fig. 12. pH dependence of the formation of ammonia from aqueous solutions of yeast cytidylic acid irradiated with 200 kv X-rays, in the presence of oxygen (1 atm.). Yeast cytidylic acid soln., 0·05%, w/v; total dose, $9{\cdot}45 \times 10^4$r units.

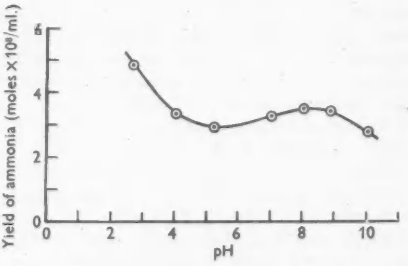

Fig. 13. pH dependence of the formation of ammonia from aqueous solutions of RNA (BYC) irradiated with 200 kv X-rays in the presence of oxygen (1 atm.). RNA soln., 0·05%, w/v; total dose, $9{\cdot}45 \times 10^{-4}$r units.

This resemblance in the behaviour of the mono- and poly-nucleotides is further demonstrated by a comparison of the yields of inorganic phosphate at different pH values (Figs. 14 and 15). Here the minimum and maximum dephosphorylations occur at pH 4–5 and pH 7–8, respectively, that is in regions which are not very different from those observed in the deamination process.

Pronounced pH effects have been previously observed by Stein & Weiss (1949), and by some other authors (Dale, Davies & Gilbert, 1949; Rotherham, Todd & Whitcher, 1951) on the yields of ammonia from amino acids, and it has been suggested that this may be due to interactions of different ionic species (Stein & Weiss, 1949). With the nucleic acids and certain related lower molecular-weight compounds it is apparent that, here again, the polar nature of the molecules may be an important factor for their reactivity.

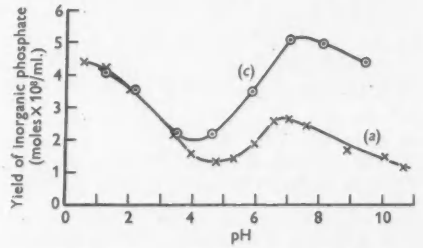

Fig. 14. pH dependence of the formation of inorganic phosphate from aqueous solutions of yeast adenylic acid (curve *a*) and yeast cytidylic acid (curve *c*) irradiated with 200 kv X-rays in the presence of oxygen (1 atm.). Soln., 0·05%, w/v; phosphate determined by method of Berenblum & Chain (1938); total dose, $9{\cdot}45 \times 10^4$ r units.

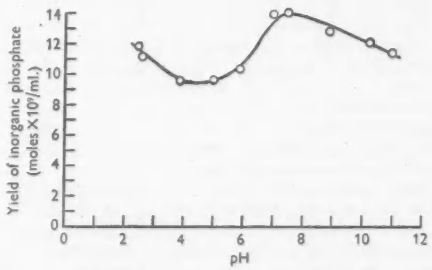

Fig. 15. pH dependence of the formation of inorganic phosphate from aqueous solutions of RNA (BY3) irradiated with 200 kv X-rays in the presence of air. RNA soln., 0·05%, w/v; Phosphate determined by the method of Berenblum & Chain (1938). Total dose, $1{\cdot}54 \times 10^5$ r units.

DISCUSSION

The nature of the chemical changes produced by the action of the radiation on the nucleic acids indicates that some of the primary linkages (i.e. glycosidic, ester, and internucleotide) are broken; thus, there appear to be various concomitant reactions, the net result of which is a fragmentation into molecular species of variable size. Owing to the complex nature of the polynucleotides, any interpretation of the mechanism of their degradation can only be regarded as tentative.

From the experimental results obtained by irradiation of the nucleosides and nucleotides (Scholes & Weiss, 1953*a*) it follows that radical attacks will occur at both the nitrogenous and carbohydrate centres of the nucleate molecule. As pointed out above, the liberation of the amino groups from adenine, guanine and cytosine should lead to a loss of hydrogen bonding and thus to a marked decrease in the structural viscosity. An attack on the pentose constituents, on the other hand, could account for the formation of free bases. Breakage of the polynucleotide chain as a result of these various reactions should lead to an increase in the number of free phosphoryl groups and this, in turn, could account for the observed increase in titratable groupings in the irradiated nucleate solutions.

One of the interesting features of the depolymerization of DNA solutions is the 'after-effect', and a somewhat similar phenomenon has also been observed in the irradiation of some RNA preparations (Grinnan & Moscher, 1951).

As pointed out above, hydrolytic processes may largely contribute to this effect and these processes may involve labile phosphate esters (see also Scholes & Weiss, 1953*b*). However, attack on the pyrimidine ethylenic bonds may also be a factor in the breakdown of the nucleic acid. It is known, for instance, that the introduction of substituents into the ethylenic centres of the pyrimidine nucleosides and nucleotides very considerably weakens the glycosidic and probably also the phosphate ester linkages. Thus bromination of uridine markedly facilitates subsequent hydrolysis by acids (Levene & La Forge, 1912). Hydrogenation of yeast cytidylic acid has also been found to enhance the rate of phosphate release by subsequent acid hydrolysis (Levene & Jorpes, 1929): this latter observation may be of some significance in view of our findings that hydrogen atoms produced by irradiation may also play a part in the dephosphorylation of the nucleic acids.

It has been stated (Butler & Conway, 1950; Conway & Butler, 1952) that the 'after-effect' in DNA solutions occurs only if the irradiations are carried out in the presence of molecular oxygen, and

these authors have suggested that the HO_2 radicals, formed under these conditions, can react with the nucleic acid forming a peroxide compound which then slowly decomposes, giving rise to the observed decrease in viscosity. If, as suggested above, the 'after-effect' is due to slow hydrolysis of labile substances formed during the irradiation process, it seems very unlikely that such compounds should be formed exclusively in the presence of molecular oxygen, as oxidizing effects can also be brought about by OH radicals which are present under conditions *in vacuo*. This has been fully confirmed by some recent experiments (Daniels, Scholes & Weiss, 1953) where it has been shown that there is also a definite 'after-effect' if the irradiations are carried out *in vacuo*: the magnitude of this effect is usually of the order of 30% of the corresponding effect in oxygenated solutions.

In the presence of oxygen, owing to the formation of hydroperoxy radicals (HO_2) according to reaction (2), back reactions such as $H + OH \rightarrow H_2O$ may be inhibited. Probably more important still, the oxidation yield may also be increased by reaction of the initially formed organic radicals (R·) with oxygen, for example

$$R\cdot + O_2 \rightarrow RO_2\cdot \rightarrow RO_2H \rightarrow \text{product}. \qquad (4)$$

It is therefore clear from the experimental results that although molecular oxygen is not the decisive factor for the 'after-effect', its presence may enhance this effect for the reasons outlined above.

This effect of oxygen is also in complete agreement with experiments on a number of other solutes, for example the formation of acetaldehyde from ethanol, pyruvic acid from lactic acid and alanine (Johnson, Scholes & Weiss, 1953), where the ratios of the yields in the presence and absence of oxygen were found to be between 3 and 6, depending on the substance and on the experimental conditions.

SUMMARY

1. Aqueous solutions of the nucleic acids have been irradiated with X-rays, using doses approaching those employed in biological experiments and the nature of the chemical changes has been investigated.

2. Quantitative determinations of the ammonia and inorganic phosphate produced by the radiation and comparison of the yields when the irradiations were performed in air, in oxygen, *in vacuo* and in hydrogen have allowed the deduction of certain features of the mechanism of attack and of the nature of the reacting species involved. In general, it has been found that the liberation of ammonia results predominantly from an attack on the constituent amino bases by the oxidative radicals OH and HO_2. In the dephosphorylation process, however, both these oxidative radicals and presumably also hydrogen atoms play an important part. Irradiations of adenine, yeast adenylic acid and yeast cytidylic acid have confirmed these views.

3. The effect of pH on the yields from these various substances has shown that, as in the case of the amino acids, the ionic nature of the substrate molecules is an important factor affecting their reactivity.

4. The decrease in viscosity observed on irradiation of DNA solutions may be attributed, at least in part, to a loss of hydrogen bonding as a result of elimination of some of the polar groups (e.g. amino or hydroxyl groups). It appears that hydrolytic processes may contribute largely to the 'after-effect' (the decrease in viscosity of nucleate solutions which occurs after cessation of irradiation) and these may involve the further slow hydrolysis of labile phosphate esters and also of compounds formed by attack on the pyrimidine ethylenic bonds.

5. Some aspects of the mode of degradation of the nucleic acids by X-rays under various experimental conditions have been discussed.

Our thanks are due to Prof. R. Signer (Berne) for the specimen of DNA, and to the North of England Council of the British Empire Cancer Campaign for financial support.

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Organic Acid Metabolism of *Penicillium chrysogenum*

1. LACTATE AND ACETATE

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The media most useful for penicillin production contain corn-steep liquor (CSL), which has a high organic acid content. Such media usually contain about 5 mg./ml. lactic acid and rather less than 0·5 mg./ml. acetic acid. It has been found beneficial to include organic acids, especially the two mentioned, in synthetic media for penicillin production (Stone & Farrell, 1946; Jarvis & Johnson, 1947; Calam & Hockenhull, 1949), and in general, media containing both acids gave better yields than those with either alone. It was considered that a study of the metabolism of these acids by *Penicillium chrysogenum* might throw light upon the biosynthesis of penicillin, and in particular upon the mode of formation of the branched-chain five-carbon skeleton of penicillamine. Dimethylpyruvic acid ($CHMe_2.CO.CO_2H$), a compound with a carbon chain similar to that of penicillamine, has been isolated from *Aspergillus niger* by Hida (1935) and, later, by Ramachandran & Walker (1951, 1952), but whereas our organism was found to produce keto acids, this acid was not isolated under parallel conditions. When lactate was used as starting material, α-oxoglutaric acid was isolated (Hockenhull, Wilkin & Winder, 1951). This work has been extended to cover other aspects of the metabolism of lactate and acetate so as to elucidate their general effects on the metabolic processes of *P. chrysogenum*.

EXPERIMENTAL

Organism. *Penicillium chrysogenum* Q176 var. *brevisterigma* described by Foster (1949) was used throughout.

Cultural conditions. These were similar to those described earlier by Hockenhull, Walker, Wilkin & Winder (1952). A sporulating culture was grown on glycerol-molasses-peptone agar slopes for 6–8 days. The spores were removed by shaking with 0·1% (v/v) aqueous Calsolene Oil HS (Imperial Chemical Industries Ltd.) to give suspensions containing 10^7–10^8 spores/ml. This suspension was transferred into 20 vol. of development medium which was incubated under standard conditions for 48 hr. Samples of this vegetative inoculum (3 ml.) were used to inoculate 60 ml. of final medium contained in 250 ml. conical flasks plugged with cotton wool. The cultures were incubated on a rotary shaker at 25°.

Media

Filtered CSL was used throughout. Removal of the sediment (comprising dead bacteria, etc.) did not significantly affect fermentation performance when this was based on the total solids content. The CSL was used on a total solids weight basis throughout ('CSL solids'), although it was added to the medium as filtered liquor containing about 40% of dissolved solids.

Development of medium. 'CSL solids' (3·8%), brown sugar (2%), phenylacetic acid (0·01%), precipitated chalk (1%), all made up (w/v) in distilled water. The pH was adjusted to 6·2 with 10N-KOH (pH after steam sterilization for 20 min. at 15 lb./sq.in. was 5·7–5·9).

CSL medium. 'CSL solids' (3·8%), lactose (3%), precipitated chalk (1%), all made up (w/v) in distilled water. No adjustment of pH was made (pH after steam sterilization for 20 min. at 15 lb./sq.in. was 5·2–5·5).

Synthetic medium. (Based on that of Jarvis & Johnson, 1947.) Lactose (2·25%), glucose (0·75%), ammonium acetate (0·3%), potassium lactate (1·0%), phenylacetic acid (0·2%), KH_2PO_4 (0·3%), $MgSO_4.7H_2O$ (0·025%), $FeSO_4.7H_2O$ (0·01%), $CuSO_4.5H_2O$ (0·0004%), $MnSO_4.4H_2O$ (0·0004%), Na_2SO_4 (0·05%), precipitated chalk (1·0%), all made up (w/v) in distilled water and adjusted to pH 6·3 with 10N-KOH before sterilization. (pH after autoclaving 20 min. at 15 lb./sq.in. steam, 5·9–6·0.)

Preparation of mycelial suspensions. Mycelium was harvested routinely at 24 hr., when the mycelial dry weight was 15–20 mg./ml. The techniques described earlier by Hockenhull *et al.* (1952) were followed by washing and starving of the mycelium. All operations were carried out aseptically.

Analytical methods

Ketonic acids. The keto acids produced were identified by paper chromatography of either the 2:4-dinitrophenylhydrazones or the nitroquinoxalinols (Hockenhull & Floodgate, 1952). As the following α-keto acids, pyruvic, oxalacetic, oxoglutaric, oxobutyric and dimethylpyruvic, might be encountered in this type of work, the methods used were tested with mixtures of the appropriate derivatives.

The 2:4-dinitrophenylhydrazones were prepared by addition of excess of a saturated solution of 2:4-dinitrophenylhydrazine in N-HCl to filtrates containing keto acids. The derivatives were then extracted with ethyl acetate, or if a copious precipitate appeared, this was filtered off, dried, and dissolved in ethyl acetate. The filtrate was extracted three times with 0·5 vol. ethyl acetate. Further purification was effected by extracting the ethyl acetate solution three

times with 0·25 vol. 5% (w/v) anhyd. Na_3PO_4, the aqueous solution was then acidified with H_2SO_4 and the 2:4-dinitrophenylhydrazones were extracted with ethyl acetate (three times with 0·125 vol.).

The nitroquinoxalinols were prepared by addition of 0·2 vol. concentrated HCl, and a calculated excess of 1:2-diamino-4-nitrobenzene as a 1% (w/v) solution in 2N-HCl. The mixtures were warmed to 60° and allowed to stand 16 hr. at room temperature. If there was any appreciable amount of precipitate, it was removed by filtration and examined separately after dissolving in ethyl acetate. The derivatives were transferred from the filtrate to ethyl acetate by extracting three times with 0·5 vol. of the solvent and the combined extracts were treated three times with 0·25 vol. 2N-NaOH. The alkaline solution of nitroquinoxalinols so obtained was acidified and the derivatives were transferred again to ethyl acetate (three extractions with 0·125 vol. solvent). The resultant solution was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure.

For the qualitative separation of the 2:4-dinitrophenylhydrazones the procedure of Cavallini, Frontali & Toschi (1949) was used with Whatman no. 4 paper. The chromatograms were developed by the ascending method with mixtures of aliphatic alcohols and dilute ammonia. The R_F values obtained are given in Table 1.

The pyruvic acid derivative gave rise to two spots, and in the early stages of this work this gave rise to some confusion. A similar phenomenon has been described by Walker, Hall & Hopton (1951), and Seligson & Shapiro (1952); it would seem probable that there are two stereoisomeric forms of pyruvic 2:4-dinitrophenylhydrazone (cf. Gordon, Wopat, Burnham & Jones, 1951). When nitroquinoxalinols were prepared from solutions giving rise to these two 'pyruvate' spots, only one derivative was obtained. A further artifact was *N*-acetyl 2:4-dinitrophenylhydrazide formed, presumably, by reaction between ethyl acetate and 2:4-dinitrophenylhydrazine. The artifact isolated after removal of the derivatives of keto acids by passage through an Al_2O_3 column. A quantity obtained from several experiments was twice recrystallized from hot aqueous ethanol as red-brown needles, m.p. 193°; the mixed melting point with authentic *N*-acetyl-2:4-dinitrophenylhydrazide (m.p. 194°) was 193–194°. (Found: C, 39·9; H, 3·6; N, 23·9. Calc. for $C_8H_8N_4O_5$: C, 40·0; H, 3·3; N, 23·3%.) This artifact was reduced to a negligible amount by keeping the temperature at which the solvent was evaporated low, by keeping the time of contact with solvent as short as possible, and by removal of excess 2:4-dinitrophenylhydrazine as soon as possible.

The nitroquinoxalinols were separated by paper chromatography (Hockenhull & Floodgate, 1952). Since carrying out this work the technique has been further improved (Hockenhull, Hunter & Herbert, 1953).

Quantitative separation of 2:4-dinitrophenylhydrazones was carried out by a method based on that of Datta, Harris & Rees (1950). A typical separation was carried out as follows. Al_2O_3 (Peter Spence and Sons Ltd., Widnes, grade O) was washed with acid by stirring in 5 vol. water and adding 2N-H_2SO_4 until the pH became constant at 4·5 (about 30 min.). It was then drained, washed with 20 vol. water, and dried in a vacuum oven at 60°. The product (25 g.) was made into a slurry with ethyl acetate and poured into a 25 mm. chromatogram tube to give a column 120 mm. long after settling. About 100 mg. of the mixed derivatives were put on to the column in about 30 ml. ethyl acetate. Development was carried out with successive 50 ml. portions of ethyl acetate : ethanol mixtures (1:0, 4:1, 1:1, v/v). A slight broadening of the band was noted with the last. Pure ethanol caused a further slight broadening of the band, but 80% (v/v) aqueous ethanol (100 ml.) washed the α-oxobutyrate and dimethylpyruvate derivatives right down the column. On the other hand, there was so much spreading that separation did not occur. The pyruvate derivative also moved in the same solvent with marked spreading and could be completely eluted with 100 ml. 50% (v/v) aqueous ethanol. The α-oxoglutarate derivative did not move. The pyruvate isomers were not separated. In an attempt to obtain sharper separations of dimethylpyruvate, α-oxobutyrate and pyruvate, 90% (v/v) aqueous ethanol containing 2% (w/v) sodium acetate ($CH_3.CO_2Na$, $3H_2O$) was employed, but, although the substances moved as compact bands, they were not separable. No suitable eluent could be found for α-oxoglutarate. This was removed, after draining the column, by extracting with 10% (w/v) Na_3PO_4. Neutral products, including *N*-acetyl-2:4-dinitrophenylhydrazide, were washed through in the ethyl acetate. Further purification of the eluates was achieved by evaporation under reduced pressure followed by recrystallization from aqueous ethanol or ethyl acetate light petroleum (b.p. 40–60°).

The nitroquinoxalinols, if they appeared homogeneous on the paper chromatogram, were purified by recrystallization. If different components were present they were separated by chromatography on Al_2O_3 (Hockenhull & Floodgate, 1952), and the fractions were recrystallized after evaporation under reduced pressure. The dimethylpyruvate and pyruvate derivatives crystallized best from hot aqueous ethanol, that of α-oxoglutarate from ethyl acetate : light petroleum (b.p. 40–60°).

Table 1. *Paper chromatography of some* 2:4-*dinitrophenylhydrazones of* α-*keto acids*

(5–10 μg. derivative in ethyl acetate spotted on to 35 mm. strips of Whatman no. 4 paper, developed in 500 ml. measuring cylinder by ascending flow of various solvents. Duration of experiments, 16 hr. Distance moved by solvent front, 24–27·5 cm. All solvents, except *sec.*-butanol, saturated with 0·8N aqueous NH_3.)

2:4-Dinitrophenylhydrazone	R_F values				
	sec.-Butanol: 0·8N-NH_4OH (4:1, v/v)	*n*-Butanol	Commercial amyl alcohol	*sec.*-Octanol	*n*-Hexanol
α-Oxoglutaric acid	0·33	0·055	0·00	0·00	0·00
Pyruvic acid (i)	0·77	0·48	0·18	0·03	0·10
Pyruvic acid (ii)	0·85	0·61	0·41	0·18	0·31
α-Oxobutyric acid	>0·95	0·76	0·56	0·30	0·47
Dimethylpyruvic acid	>0·95	0·83	0·68	0·47	0·62

Lactic acid. This was assayed either by the method of Barker & Summerson (1941) or by that of Friedemann, Cotonio & Shaffer (1927), using the apparatus of Shinn & Nicolet (1941) but, where interference from pyruvate was likely, a modification of the ceric sulphate oxidation method of Gordon & Quastel (1939) was used. The methods agreed well. Lactic acid (90%), B.P., was used throughout.

Reducing sugars. These were determined by the method of Shaffer & Hartmann (1920) after hydrolysis in 0·8 N-HCl for 30 min. at 15 lb. steam/sq.in.

Acetic and other volatile acids. These were determined by the method of Friedemann (1938) using the apparatus as modified by Hockenhull (1949). The acids were distilled in a current of steam after acidification and addition of sodium tungstate and $MgSO_4$, $7H_2O$. The distillate was decarbonated by passing CO_2-free air, and then titrated with CO_2-free alkali using phenol red as indicator. Determination of the partition coefficient of the volatile acids between ether and water indicated that, in the absence of phenylacetic acid or similar benzyl precursors acetic acid was the only volatile acid present in culture filtrate. Glacial acetic acid (A.R.) was used throughout.

Manometric measurements. These were carried out in a standard type Warburg apparatus (Hospital and Laboratory Supplies Ltd., London, E.C. 1), using 20 ml. cups with centre wells and single side bulbs. All observations were made at 25°. $Q_{O_2} = \mu$l. O_2/mg. dry weight/hr.

RESULTS

Lactate metabolism

Lactate utilization by growing cultures. Replicate cultures on synthetic medium were harvested daily in triplicate. The sugar and lactate contents of the media were determined, and the mycelium was used for estimation of the Q_{O_2} with lactate as the substrate (Fig. 1). Cultures grown on CSL medium were also examined (Fig. 2).

Lactate disappeared extremely slowly during the first 96 hr. from both media; this was in marked contrast with the results obtained by Jarvis & Johnson (1947), with the parent strain *P. chrysogenum* Q. 176. In CSL medium, however, there was a marked increase in rate of lactate disappearance after 96 hr. At this time, the sugar level had fallen to about 40% of the initial value. This increased rate of lactate utilization appeared to coincide roughly with a rapid rise in penicillin titre. On the other hand, in synthetic medium the rate of lactate disappearance remained extremely slow for the full 120 hr. growth period.

Examination of the Q_{O_2} (lactate) values of washed, starved mycelium from the synthetic medium, showed that the lactate disappearance in growing cultures (2 mg./ml. over 120 hr.) was far less than that calculated from the oxygen usage by washed suspensions taken at intervals over this period (about 28 mg./ml.). Assuming that the washing and starving techniques had not increased the ability of mycelium to use lactate, it appeared

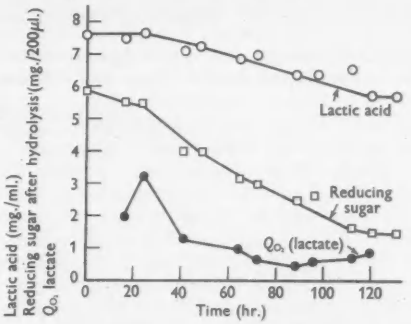

Fig. 1. Disappearance of lactate in synthetic medium. Shaken cultures at 25°. 60 ml. synthetic medium/250 ml. flask. Triplicate flasks removed at intervals for analysis. ○—○, lactic acid; □—□, reducing sugar after hydrolysis; ●—●, Q_{O_2} (lactate) values given by mycelial sample after washing and starving according to standard technique at 25°.

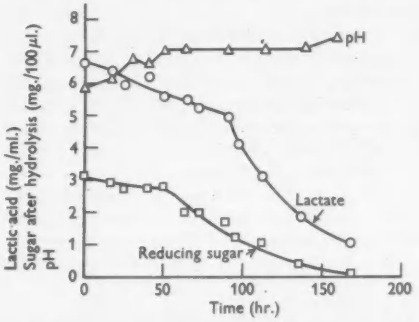

Fig. 2. Disappearance of lactate in CSL medium. Shaken cultures at 25°. 60 ml. CSL medium/250 ml. flask. Triplicate flasks removed at intervals for analysis. ○—○, lactic acid; □—□, reducing sugar after hydrolysis; △—△, pH values.

that some factors slowing up the utilization of lactate were present in the medium.

Effect of glucose upon lactate utilization. An experiment was carried out using mycelium washed and starved in 0·1 M potassium phosphate (pH 7·3) according to the standard technique. This was washed 3 times and suspended in 0·1 M potassium phosphate (pH 5·0). Samples (20 ml.) of this suspension were measured into 250 ml. conical flasks and followed by 10 ml. of the pH 5·0 buffer containing appropriate amounts of the substrates. The flasks were shaken aerobically at 25° after withdrawal of a 5 ml. homogeneous sample for analysis. After 6 hr. the reaction mixtures were again sampled. The two sets of samples were filtered and the amounts of lactate determined (Table 2).

Table 2. *Effect of glucose on the lactate-pyruvate system*

(25 ml. suspension (about 15 mg./ml. dry weight) on 0·1 M potassium phosphate, pH 5·0 and containing added substrates neutralized with KOH placed in 250 ml. conical flasks and shaken 6 hr. at 25°. Lactate determinations at 0 and 6 hr.)

	Substrates added				
Expt.	Lactic acid (4 mg./ml.)	Pyruvic acid (4mg./l.)	Glucose (40 mg./ml.)	Utilization of lactic acid (mg/ml.)	Average (mg./ml.)
1	+	−	−	1·54 2·02 2·02 1·92	1·87
1	+	−	+	0·54 0·31 0·11 0·33	0·32
2	+	−	−	0·17* 0·70 1·01	0·63
2	+	+	−	0·75 0·89 0·12	0·59
2	+	−	+	0·05 0·37 0·25	0·22
2	−	+	−	0·00 0·01 0·02	0·01
2	−	+	+	−0·26* −0·20 −0·32	−0·26
2	−	−	+	−0·05 −0·07 +0·15	0·01

* Note low value for lactic acid utilization.

Although, in absence of glucose, there was sometimes a very slow utilization of lactate (one result marked * in Table 2), it is clear that in general glucose suppressed lactate utilization, while it did not give rise to lactate on its own. Glucose, however, caused the reduction of pyruvate to lactate. Washed, starved mycelium would not oxidize lactate anaerobically with either methylene blue, resazurin or potassium ferricyanide (Quastel & Wheatley, 1938) as acceptors. All subsequent respirometric studies were carried out aerobically (in air).

Effect of hydrogen-ion concentration on oxygen uptake. Washed, starved suspensions were prepared according to the standard technique. Starving was carried out in 0·1 M potassium phosphate at pH 7·3 and after washing re-suspended in buffer at the pH to be investigated. The Q_{O_2} (lactate) values were determined over 30 min. in Warburg manometers. Fig. 3 shows the relation of activity to pH.

Effect of substrate concentration on oxygen uptake. Washed, starved suspensions at pH 7·3 were used.

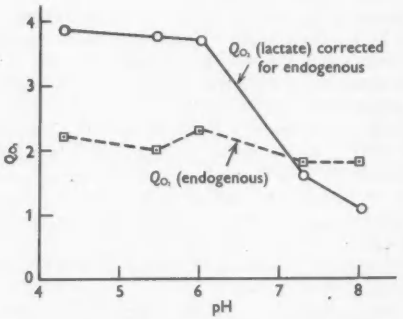

Fig. 3. Effect of hydrogen-ion concentration upon Q_{O_2} (lactate) values. Warburg flasks. Main compartment: 2 ml. washed, starved suspension (30 mg. dry wt.) in 0·1 M potassium phosphate at appropriate pH. Side bulb: 0·3 ml. 0·1 M potassium lactate at appropriate pH. In centre well: 0·2 ml. 10 N-KOH on filter paper, 25°. Air. ○—○, Q_{O_2} (lactate) corrected for endogenous respiration; ⊡—⊡, Q_{O_2} (endogenous) controls.

Their Q_{O_2} (lactate) were measured in Warburg manometers against various concentrations of lactate over 30 min. The curve obtained (Fig. 4) was somewhat shallower than the typical Michaelis curve. This appeared to be due to further metabolism of pyruvate and was probably further complicated by exhaustion of substate which made it difficult to estimate accurately the initial velocity at the lower concentration. The lowest concentration of lactate used (0·0001 M) gave a total oxygen consumption of 2·8 moles/mole lactate. Complete oxidation to carbon dioxide required 3 moles.

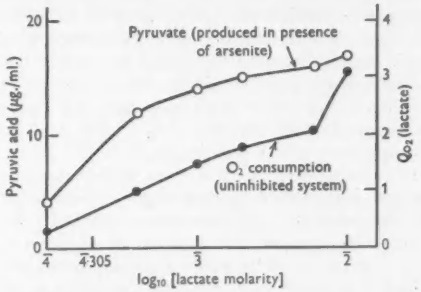

Fig. 4. Effect of substrate concentration upon Q_{O_2} (lactate). Warburg flasks. Main compartment: 2 ml. washed, starved mycelial suspension (about 30 mg.) in 0·1 M potassium phosphate buffer pH 7·3. Side bulb: 1 ml. potassium lactate at appropriate concentration (10^{-5}–10^{-2} M). In centre well: 0·2 ml. 10 N-KOH on filter paper, 25°. Air. 'K_m'$=2\text{ M}\times10^{-4}$. All figures were corrected for endogenous respiration or production of pyruvate. The latter correction was negligible.

The same experiment was repeated in presence of arsenite. Owing to the inhibition of the oxidation of pyruvate by arsenite, the amount of oxygen consumed was reduced to one-sixth, which was below the limit of accuracy of the manometric method. Accordingly, the amount of keto acid formed in 20 min. was measured using the method of Friedemann & Haugen (1943). When the amount of keto acid produced was plotted against substrate concentration, a Michaelis-type curve ($K_m=2\times10^{-4}$ M) was obtained (Fig. 4). It was confirmed by paper chromatography that the substance measured was pyruvate. It must be emphasized that the Michaelis constant is merely a convenient symbol, and does not have the same meaning for whole cells as that defined originally for isolated enzymes.

Effect of inhibitors on oxygen uptake. The oxygen uptake of suspensions of *P. chrysogenum* at pH 7·3 prepared in the usual way was studied in presence of various inhibitors. Arsenite (0·01 M) and iodoacetate (0·001 M) showed considerable inhibition. On the other hand, 2:4-dinitrophenol had no effect on the oxygen uptake in the presence of substrate, although it increased the endogenous respiration (Table 3). Malonate (0·01 M) inhibited lactate oxidation at neither pH 5·2 nor 7·2. Streptomycin (0·001 M) and methylene blue (0·001 M) were also found to have no effect. Arsenite (0·01 M) and iodoacetate (0·001 M) were found to inhibit oxidation of pyruvate.

Products obtained by aerobic metabolism of lactate. Keto acids accumulated when washed, starved mycelium, suspended in 0·1 M potassium phosphate, pH 7·0–7·5, was incubated with lactate aerobically

Table 3. *Effect of inhibitors on* Q_{O_2} *(lactate) and* Q_{O_2} *(pyruvate) values*

(Warburg manometers. In main vessel, washed, starved mycelial suspension (25–30 mg. dry wt.) in 0·1 M potassium phosphate, pH 7·3, 0·3 ml. 0·1 M potassium lactate or potassium pyruvate, 0·3 ml. 0·1 M potassium phosphate. In side bulb, 0·3 ml. 0·1 M potassium phosphate containing inhibitor. In centre well, 0·1 ml. 10 N-KOH on filter paper. Respiration measured over 20 min. at 25° in air.)

			Q_{O_2} values				
Substrate	Inhibitor	Concentration (M)	Control	With inhibitor	With substrate	With substrate and inhibitor	Inhibition (%)
Potassium lactate	Sodium arsenite	0·01	2·95 2·95	2·15 2·10	11·80 11·40	3·55 3·25	85
Potassium lactate	Sodium arsenite	0·01	2·05	1·25	3·80	1·30	95
Potassium lactate	2:4-Dinitrophenol	0·001	2·05	2·40 2·30	3·80	4·25 4·00	0
Potassium lactate	Potassium iodoacetate	0·001	2·15 1·85	1·25 1·15	2·95 2·85	1·15 1·10	100
Potassium pyruvate	Sodium arsenite	0·01	3·50 3·25	1·60 1·50	4·80 4·55	2·00 1·95	55
Potassium pyruvate	Potassium iodoacetate	0·001	2·70 2·85	2·10 2·20	4·25 4·20	2·15 2·15	100

for 10–24 hr. in the presence of either 0·01 M sodium arsenite or 0·001 M 2:4-dinitrophenol. For an extended period, during which most of the reported results were obtained, the yields of keto acids accounted for 20–40 % of the lactate carbon supplied. However, after an interval of some months, when it was desired to check and extend certain observations, great difficulty was encountered in getting yields even as high as 5–10 %, although the procedure was exactly the same. Finally, for no apparent reason, yields of the order of 20 % were again encountered. This variation, which was not susceptible to our direct control, might have been due to such phenomena as differences in the nitrogen retention of the mycelium, or the leaching of essential substances during starvation, or even to the previous mycological history of the organism.

With all treatments, both pyruvate and α-oxoglutarate were obtained in varying ratios from 0 to 100 %. Again these were not predictable.

The accumulation of α-oxoglutarate in presence of arsenite is in apparent contradiction to the results quoted in the substrate concentration experiment (Fig. 4) though here conditions were very different (incubation time 24 hr. as against 30 min.; substrate concentration 0·11 M as against 0·01 M).

Metabolic products obtained at pH 5·0. The isolation and characterization of the 2:4-dinitrophenylhydrazone of α-oxoglutaric acid under these conditions have been described already (Hockenhull *et al.* 1951). Similar experiments yielded pyruvic acid almost exclusively. This was characterized as both the 2:4-dinitrophenylhydrazone and as the 3-methylnitroquinoxalin-2-ol. Washed, starved suspensions from 60 ml. of broth prepared under the standard conditions and re-suspended in 60 ml. 0·1 M potassium phosphate of pH 5·0 (about 15 mg./ml. dry weight) were shaken at 25° in 250 ml. flasks for 24 hr. Reaction mixtures from three such flasks were filtered and treated with about 1 g. 1:2-diamino-4-nitrobenzene; the product which was mainly the derivative of pyruvate was purified as described above, and recrystallized from hot aqueous ethanol to yield about 300 mg., m.p. 254–255° (authentic 3-methylnitroquinoxalin-2-ol, 255–6°, mixed m.p. 254–255° (uncorr.). (Found: C, 52·1; H, 3·3; N, 20·9. Calc. for $C_9H_7N_3O_3$: C, 52·7; H, 3·4; N, 20·5 %.)

Metabolic products obtained in presence of arsenite. A washed, starved, mycelial suspension in 0·1 M potassium phosphate of pH 7·4 (15 mg./ml. dry weight) was prepared from an equal volume of 24 hr. culture on synthetic medium. To this was added 10 mg./ml. lactic acid as the potassium salt, and sodium arsenite to a final concentration of 0·01 M. The suspensions were shaken in 250 ml. flasks, 60 ml. in each, for 24 hr. at 25°, and then filtered. From 280 ml. of filtrate (five flasks), 400 mg. of crude 2:4-dinitrophenylhydrazone were isolated. This was almost wholly the derivative of α-oxoglutarate. Its characterization is described elsewhere (Hockenhull *et al.* 1951).

Other experiments yielded pyruvate equivalent to 30–40 % of the lactate carbon supplied.

Metabolic products obtained in presence of 2:4-dinitrophenol. An experiment similar to the above but in which 0·001 M dinitrophenol was used instead of arsenite yielded about 1·0 g. of 2:4-dinitrophenylhydrazones from eight flasks. The mixed 2:4-dinitrophenylhydrazones were separated on an alumina column and the α-oxoglutarate derivative was isolated. Recrystallization of the product gave three fractions weighing 142, 359 and 202 mg. respectively. These melted between 212 and 215°. After being pooled and recrystallizing from ethyl acetate : light petroleum, a pure product m.p. 218–219°, giving no depression of melting point with authentic material (m.p. 219°), was obtained.

In a similar experiment 800 mg. of impure dinitrophenylhydrazones consisting largely (about 80 %) of the derivative of pyruvate were obtained. After purification by chromatography on alumina and recrystallization of the 450 mg. of derivative in the eluate, a product, m.p. 217–218° (mixed m.p. 218° with authentic material), was isolated.

Acetate metabolism

Acetate utilization by growing cultures. Replicate cultures on synthetic medium were harvested daily in triplicate and the amounts of acetate determined. It was found that the acetate had almost completely disappeared by the time that the sugar level had fallen to some 50 % of its initial value (Fig. 5). Similar behaviour was shown in CSL medium as recorded in a previous paper (Hockenhull *et al.* 1952, Fig. 1).

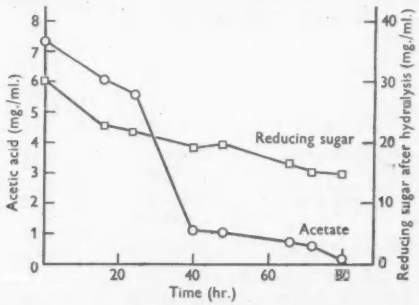

Fig. 5. Disappearance of acetate in synthetic medium. Shaken cultures at 25°. 60 ml. synthetic medium/250 ml. flask. Triplicate flasks removed at intervals for analysis. ○—○, Acetic acid; □—□, reducing sugars after hydrolysis.

Effect of hydrogen-ion concentration on oxygen uptake. Washed, starved suspensions were prepared according to the standard techniques. Starving was carried out at pH 7·3 with subsequent washing and re-suspension in buffer at the pH value to be investigated. The Q_{O_2} values were determined during 30 min. in Warburg manometers. Fig. 6 shows the relation of activity to pH. The optimum lay between pH 6 and 7.

Effect of substrate concentration on oxygen uptake. Washed, starved suspensions prepared according to the standard technique at pH 7·0 throughout were used. The Q_{O_2} values were determined in Warburg manometers over 15 min. with different levels of potassium acetate. A curve, resembling in form the typical Michaelis curve for a single enzyme, was obtained. The concentration at which half maximum activity was shown was higher than might be expected (0·0012 M) (Fig. 7).

Effect of inhibitors on oxygen uptake. Marked inhibition occurred in presence of arsenite, iodoacetate or sodium azide (Table 4); on the other hand, malonate (0·01 M), streptomycin (0·001 M) and methylene blue (0·001 M) were ineffective as inhibitors. Calcium ions (0·1 M), added to trap oxalate, did not affect the amount of oxygen taken up in presence of acetate. This implied that acetate was not oxidized to carbon dioxide by way of oxalate.

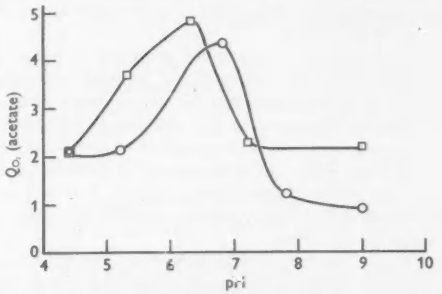

Fig. 6. Effect of hydrogen-ion concentration upon Q_{O_2} (acetate) values. Warburg flasks. Main compartment: 2 ml. washed, starved mycelial suspension (30 mg. dry wt.) in 0·1 M potassium phosphate buffer at appropriate pH, 0·7 ml. water. 0·3 ml. 0·1 M potassium acetate at appropriate pH. In centre well: 0·2 ml. 10 N-KOH on filter paper. ○—○ and □—□ represent two similar experiments both at 25° in air.

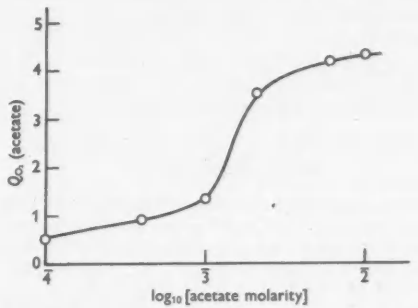

Fig. 7. Effect of substrate concentration upon Q_{O_2} (acetate) values. Warburg flasks. Main compartment: 2 ml. washed, starved mycelial suspension (30 mg. dry wt.) in 0·1 M potassium phosphate buffer pH 7·3. Side bulb: 1 ml. potassium acetate, pH 7·3, at appropriate concentration (10^{-5} – 10^{-2} M). In centre well: 0·2 ml. 10 N-KOH on filter paper, 25°. Air. 'K_m' = 1·2 M × 10^{-3}.

Table 4 *Effect of inhibitors on* Q_{O_2} *(acetate) values*

(Warburg manometers. In main vessel, washed, starved mycelial suspension (25–30 mg. dry wt.) in 0·1 M potassium phosphate, pH 7·3, 0·3 ml. 0·1 M potassium acetate, 0·3 ml. 0·1 M potassium phosphate. In side bulb, 0·3 ml. 0·1 M potassium phosphate containing inhibitor. In centre well, 0·1 ml. 10 N-KOH on filter paper. Oxygen uptake measured over 20 min. at 25° in air.)

			Q_{O_2} (acetate) values				
Expt.	Inhibitor	Concentration (M)	Control	With inhibitor	With substrate	With substrate and inhibitor	Inhibition (%)
1	Sodium arsenite	0·01	2·5 2·40	1·10 1·10	6·30 6·00 6·10	1·10 1·10	100
2	Potassium iodoacetate	0·001	2·60 2·55	2·40 2·45	6·00 5·80	2·40 2·50	100
3	Potassium iodoacetate	0·01 0·001 0·0001 0·00001	1·45	1·80 1·50 1·50 1·40	2·55	1·75 1·70 1·70 2·85	100 80 80 −30
4	NaN_3	0·003	2·55 2·30	2·75 2·55	5·30 5·40	3·60 3·55	70

Products obtained by aerobic metabolism of acetate and other acids related to the tricarboxylic acid cycle. Attempts were next made to demonstrate the formation of ketonic acids from acetate using the three sets of conditions under which these compounds were produced from lactate. Very small amounts of keto acids were produced from acetate, although parallel experiments with lactate as the substrate gave high yields.

Similar experiments were carried out with certain of the intermediates of the tricarboxylic acid cycle and related compounds. Portions of mycelial suspensions at pH 5·0 were incubated with lactate, acetate, malate, fumarate, succinate, glutamate and citrate, respectively. The amount of substrate (neutralized with potassium hydroxide) was adjusted to give 4 g. carbon/l. (equivalent to 1 %, w/v, lactic acid). Incubation was carried out in 60 ml. flasks as described above. The keto acids were converted into 2:4-dinitrophenylhydrazones, which were then treated as described on p. 74. The final solution in ethyl acetate was dried over anhydrous sodium sulphate and then evaporated to dryness and the product was weighed. Table 5 gives the weights of total 2:4-dinitrophenylhydrazones produced from the various substrates. A rough quantitative estimate of the relative amounts of pyruvate and α-oxoglutarate was made by paper chromatography. The estimate was made by visual comparison with standard mixtures, and the precision was about ± 10 %. The experiment quoted (Table 5) was typical, but in view of the variability sometimes encountered, for example with lactate, caution must be exercised in the interpretation of these results. When glucose or lactose at comparable levels were used as substrate there was no appreciable accumulation of keto acids.

Table 5. *Production of ketonic products from various organic acids*

(24 hr. incubation at 25° with shaking of 250 ml. conical flask containing 60 ml. mycelial suspension (25–30 mg./ml. dry wt.) in 0·1 M potassium phosphate, pH 5·0, together with organic acid neutralized with 10 N-KOH at a carbon level corresponding to 1 % lactic acid.)

Substrate	Yield of 2:4-dinitrophenylhydrazones equivalent to 12 g. original C. (g.)	Approx. proportions of constituents: α-Oxoglutarate (%)	Pyruvate (%)
Lactate	18·8	30	70
No addition	Negligible	.	.
Acetate	2·3	0	100
Malate	6·6	40	60
Fumarate	9·9	70	30
Succinate	5·3	100	0
Glutamate	1·6	100	0
Citrate	Negligible	.	.

Table 6. Q_{O_2} *values for members of the tricarboxylic acid cycle*

(Warburg manometers. In main vessel: washed, starved mycelial suspension (about 30 mg. dry wt.) in 2·7 ml. 0·1 M potassium phosphate, pH 7·3. In side bulb, substrate pH 7·3 at 0·1 M. In centre well, 0·1 ml. 10 N-KOH on filter paper. Oxygen uptake measured over 20 min. at 25° in air.)

Substrate	Q_{O_2} (total) (uncorrected)	Q_{O_2} corrected for endogenous respiration
Acetate	6·10	3·40
Lactate	3·80	1·10
Succinate	5·55, 5·50	2·85, 2·80
Fumarate	4·65, 4·70	1·90, 2·00
Malate	6·20, 6·70	3·50, 3·40
Citrate	3·70, 3·40	0·95, 0·65
None	2·75,* 2·70*	0, 0

* Endogenous respiration.

The oxygen consumption of washed, starved mycelium in the presence of acetate, lactate, fumarate, malate and citrate was measured, and the results, expressed as Q_{O_2}, are given in Table 6. The Q_{O_2} was smallest in the presence of citrate, which might imply that the conversion of this compound into α-oxoglutarate was slow. It must, however, be emphasized that the Q_{O_2} values varied from preparation to preparation.

Effect of incubation with acetate and lactate on the dry weight of the mycelium. Washed, starved mycelium suspended in phosphate buffer of pH 7·4 was incubated for 24 hr. with either acetate or lactate, and the dry weights of representative samples of mycelium were determined at the beginning and end of the experiment. The results are shown in Table 7. It will be seen that, in the absence of substrate, the dry weight fell over the 24 hr. period, and that addition of acetate increased this loss of weight. In contrast, incubation with lactate prevented any loss of weight. Inclusion of either cyanide, semicarbazide, 2:4-dinitrophenol or arsenite in the incubation mixture had little effect, although there was a slight loss of weight in the presence of the last two when lactate was the substrate.

Identification of tricarboxylic acid-cycle intermediates in the mycelium. Mycelium grown on 1 vol. CSL medium was harvested at 48 hr. It was spun off, washed 3 times in 0·1 M potassium phosphate buffer, pH 7·3, re-suspended in 0·5 vol. of this buffer, and 0·5 vol. of a simplified double-strength medium pH 6·5 (lactose 4 %, ammonium acetate 0·75 %, precipitated chalk 1 %, KH_2PO_4 0·1 %, all w/v) was added aseptically. This suspension was incubated under standard conditions (60 ml./250 ml. conical flask) at 25° for 24 or 66 hr.

At the end of the chosen period the mycelium was filtered off and extracted with hot 50 % (v/v) aqueous ethanol (5 ml./flask). The extract so obtained was studied by descending chromatography on Whatman no. 4 paper with the following solvent mixtures: *n*-butanol:formic acid:water (4:1:5, v/v), mesityl oxide:formic acid:water (1:1:2, v/v), and *iso*propanol:*tert.*-butanol:benzyl alcohol:formic acid:

Table 7. *Effect of lactate or acetate upon mycelial weight*

(250 ml. shake flasks containing about 15 mg./ml. mycelium harvested at 24 hr. suspended in 0·1 M potassium phosphate, pH 7·3, containing either 1 % acetic acid or 1 % lactic acid as K salts, together with additional trapping agents or inhibitors as indicated. Incubated 24 hr. with shaking at 25°.)

	Mycelial dry weight (g./flask)			
			Final (24 hr.)	
Addition	Initial (0 hr.)	Control	Acetic acid 1 %	Lactic acid 1 %
None	0·88	0·65	0·47 (72)*	0·82 (126)
None	0·93	0·73	0·52 (71)	0·80 (110)
0·01 M-KCN	0·88	0·65	0·49 (76)	0·92 (142)
0·01 M Semicarbazide	0·88	0·60	0·45 (75)	0·83 (138)
0·001 M 2:4-Dinitrophenol	0·88	0·67	0·51 (76)	0·72 (108)
0·01 M Sodium arsenite	0·93	0·74	0·51 (69)	0·75 (102)

* Figures in parentheses represent weight as percentage of final control.

water (10:10:30:2:10, v/v) and the aqueous layers were discarded after equilibration. The techniques were similar to those described by Bryant & Overell (1951), and Stark, Goodman & Owens (1951). They suffered from the disadvantage that lactate and succinate were not sharply separable.

The following acids were identified after 24 hr. incubation: glutamic acid (0·13 mg.), citric acid (0·10 mg.), malic acid (0·80 mg.), lactic or succinic acid (0·05 mg. as lactate), and fumaric acid (0·03 mg.). The quantities given were rough estimates (± 25 %) of the total quantities of acid produced in the felt from 60 ml. of original broth. Qualitatively similar results were obtained after 66 hr. incubation. The extract gave a positive reaction with sulphuric acid and ethanolic guaiacol, which suggested the presence of lactate.

Fermentations carried out using radioactive acetate. In an experiment designed for the production of penicillin containing radioactive carbon (to be described later), a medium (Calam & Hockenhull, 1949) was made up to contain $^{14}CH_3COOH$ (0·33 μc/mg.). After the growth of *P. chrysogenum* under standard cultural conditions for 6 days at 25°, the specific activity of the acetate fell from 0·33 to 0·0046 μc/mg. This large dilution indicated that a considerable amount of acetate was produced by the mould. During the course of the experiment the acetate concentration fell from 2·5 to 0·20 mg./ml.

DISCUSSION

Glucose has been found to suppress lactate utilization by washed, starved mycelium and *P. chrysogenum*, and when glucose was added to such preparations in the presence of pyruvate, lactate was produced. It is interesting that this effect was observed under the highly aerated conditions obtained in shake-flask cultures.

Lactate is oxidized to pyruvate, and this step does not appear to be carried out unless oxygen is supplied. Electron acceptors such as ferricyanide and methylene blue were ineffective. α-Oxoglutarate has been shown to be formed from lactate.

The most likely catabolic pathways open to pyruvate (and hence to lactate) are (*a*) oxidation to 'active acetate' and carbon dioxide, and (*b*) carboxylation by a Wood-Werkman type of reaction (Wood & Werkman, 1938), or one involving the malic enzyme of Ochoa, Mehler & Kornberg (1948). Further oxidation of 'active acetate' is known to proceed, at least in animal tissues, by the tricarboxylic acid cycle. This requires to prime it a supply of C_4-dicarboxylic acids, a small amount of these acids being all that is required for continuous operation of the cycle. If, however, there is, at the same time, a synthesis of C_4-acids from part of the pyruvate, it is not unreasonable to expect an accumulation of components of the cycle such as α-oxoglutarate. On the other hand, unless acetate itself can give rise to C_4 acids, no accumulation of α-oxoglutarate is to be expected, for, if there were, this would result in the locking up of the small basal amount of C_4 acids, with the result that the cycle would come to a stop. This clearly does not happen with acetate which is metabolized rapidly. It is possible that there is some wastage of C_4 acids, as they participate in the cyclic oxidation of acetate. If these are replenished from the mycelial contents, the increased mycelial wastage in presence of acetate might in some part be explained.

There is no reason to suppose that, in *P. chrysogenum*, acetate is condensed to a C_4 compound by a process such as that depicted by Ajl (1951) in his Fig. 2. This deficiency may be a specific property of the organism, or it may be due to lack of activation energy, for it has been reported by Foster *et al.* (1949, 1950) that the synthesis of fumarate from acetate is only 10–15 % of that from a similar molar concentration of ethanol. It is improbable that any significant amount of acetate is oxidized directly by way of oxalic acid.

Attention is drawn to differences in the relationships between substrate concentration and enzyme activity for the two substrates, especially with respect to the acetate curve. While it is not in order to base any reasoning about mechanisms upon this type of evidence, there is nothing in the data to contradict the above hypotheses.

The high degree of dilution of radioactive acetate by the mould points to the internal production of acetate and its presence as a normal participant in mould metabolism. Acetate has been detected in cultures of *Aspergillus niger* (Chughtai, Pearce & Walker, 1947), and *A. nidulans* (Hockenhull, 1950), when glucose was the sole carbon source.

SUMMARY

1. Lactate and acetate are oxidized by growing cultures and washed, starved mycelial suspensions of *Penicillium chrysogenum*.

2. Mycelial suspensions oxidize lactate only in the presence of oxygen, and the optimum pH is in the range 4·0 to 6·0. In the presence of arsenite, half maximum velocity of lactate oxidation occurs at a substrate concentration of 2×10^{-4} M.

3. The first product of lactate oxidation is pyruvate. This is reduced back to lactate in presence of glucose. Lactate is not formed from glucose alone.

4. The Q_{O_2} (lactate) was inhibited by both arsenite (0·01 M) and iodoacetate (0·001 M); these compounds also inhibited the Q_{O_2} (pyruvate). Malonate, streptomycin, methylene blue or 2:4-dinitrophenol had no effect on the Q_{O_2} (lactate).

5. Prolonged aerobic incubation of mycelium with lactate under specified conditions leads to the accumulation of considerable amounts of pyruvate and α-oxoglutarate. Acetate, citrate and glutamate under the same conditions give very low yields of ketonic products. Succinate, malate and fumarate give intermediate amounts of keto acids, all much less than lactate. All these acids increase oxygen uptake by mould mycelium.

6. Acetate utilization, unlike that of lactate, is rapid in presence of glucose.

7. Acetate oxidation by mycelium is maximal between pH 6 and 7. The rate of oxidation of acetate is half maximum at an acetate concentration of $1·2 \times 10^{-3}$ M.

8. The Q_{O_2} (acetate) is depressed by arsenite (0·01 M), iodoacetate (0·001 M), and azide (0·003 M). Malonate, streptomycin and methylene blue do not inhibit.

9. Acetate causes mycelium to 'waste' on prolonged incubation, whereas lactate tends to conserve mycelial dry matter.

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The Fission of Tobacco Mosaic Virus and some other Nucleoproteins by Strontium Nitrate

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During the first few years of work on the chemical properties of preparations of tobacco mosaic virus, the view that activity was associated with a nucleoprotein was contested; this led to an examination of the conditions under which the nucleic acid and the protein in a virus preparation remained associated or were separated. As a rule the presence of a link was assessed by seeing whether the protein and nucleic acid sedimented together on ultracentrifugation and by the separation of a phosphorus-free coagulum of protein. The linkage was not apparently broken by high concentrations of the salts generally used in protein fractionation, e.g. sodium chloride and ammonium sulphate, nor by exposure for a few hours at room temperature between pH's 0 and 9. Separation was, however, easily effected by the traditional method of exposure to cold alkali, and also by boiling in dilute, neutral salt solutions, by running the nucleoprotein solution into several volumes of acetic acid or by treatment with pyridine (Bawden & Pirie, 1937). Sreenivasaya & Pirie (1938) separated the protein and nucleic acid by incubating virus preparations with 10 g./l. solutions of sodium dodecyl sulphate; this agent was used by Bawden & Pirie (1940*b*), who also got separation with concentrated phenol and various other substances, including urea (Bawden & Pirie, 1940*a*). These observations indicate that, very probably, the preparations studied are nucleoproteins rather than protein nucleates. That is to say, the linkage between the protein and nucleic acid is not a readily dissociable salt linkage, but only breaks when one or other partner is modified. The protein moiety, for example, is denatured when the virus is heated or dissociated by such agents as urea.

This conclusion gave an added interest to the observation that tobacco mosaic virus preparations dissociate when exposed for a few hours to molar solutions of strontium nitrate. No detailed study has been made because this is not the type of phenomenon with which we are primarily concerned; some rather casual observations on it may, however, be reported.

EXPERIMENTAL

Experiments with tobacco mosaic virus preparations. During a 10-year period, many different virus preparations have been used. Some were aggregated preparations made by precipitation with acid and $(NH_4)_2SO_4$ (Bawden & Pirie, 1943*a*) and some were fractions, with differing degrees of aggregation, made by differential ultracentrifugation (Bawden & Pirie, 1945). The two types of preparation behaved similarly with $Sr(NO_3)_2$. All preparations had lain for some months in a refrigerator and were ultracentrifuged again immediately before use. The preparations made by ultracentrifuging only are similar to those normally used by other workers on this virus, and although their properties differ widely from those of the bulk of the anomalous nucleoprotein in the infected leaf, they will for convenience be referred to as virus or *TMV*.

Solutions containing *TMV* and $Sr(NO_3)_2$ are initially clear and show the anisotropy of flow characteristic of *TMV* solutions. After an interval, which depends on the pH, the temperature, and the concentrations, the anisotropy of flow diminishes, an opalescence appears, and later a precipitate. The course of one such experiment is shown in Table 1. The separation of nucleic acid in this system, like the separation by boiling, is accompanied by a fall in pH; the borate buffer present is only sufficient to keep the fall from exceeding 1 unit. When the concentration of $Sr(NO_3)_2$ is smaller, or that of *TMV* greater, the precipitate that forms has a different texture from that separating under the conditions of Table 1. The latter is dense and centrifuges out easily, whereas the former compacts badly and, at least when newly formed, disperses completely when vigorously shaken, but re-forms on standing. This behaviour has been

Table 1. *Effect of* pH *on the fission of TMV by* $Sr(NO_3)_2$

(Solutions containing (final concentrations) 3·5 g./l. *TMV* and 1·6 M-Sr $(NO_3)_2$ adjusted to the pH stated and made 0·03 M with sodium borate buffer of the same pH. Kept at 21° and the appearance noted at intervals.)

	Time				
Initial pH	10 min.	30 min.	2 hr.	4 hr.	20 hr.
8·0	No change	No change	Opal.	Opal.	Ppt.
8·5	Faint opal.	Opal.	Ppt.	—	—
9·0	Opal.	Opal.	Ppt.	—	—
9·5	Ppt.	—	—	—	—

noticed in other systems containing *TMV* both *in vivo* and *in vitro* (cf. Bawden & Pirie, 1940*b*). In this system it is encountered with $Sr(NO_3)_2$ weaker than M and *TMV* stronger than 15 g./l.

The concentrations affect not only the physical character of the precipitate but also its composition; the dense type of precipitate is substantially free from nucleic acid, whereas that separating from concentrated *TMV* or dilute $Sr(NO_3)_2$ solutions carries much or all of the nucleic acid with it. Separation can be brought about by adding alkali, but not by a further few weeks of exposure to dilute $Sr(NO_3)_2$. A mixture that has precipitated without fission because of an inadequate concentration of $Sr(NO_3)_2$ does not undergo fission consistently if the concentration is increased later. Similarly, complete fission cannot be obtained under these conditions and with a final virus concentration greater than 15 g./l. by adding the virus gradually during several hours.

This system offers a method for preparing nucleic acid from *TMV* which seems to expose the nucleic acid to conditions less drastic than those generally used. In any study of the original state of the nucleic acid in *TMV* it may therefore prove useful.

Nucleic acid made in this way has the usual resinous texture when precipitated with acid. It is readily attacked by both pancreatic and tobacco-leaf ribonuclease, and, like preparations made in other ways from *TMV*, it is relatively ineffective as an inhibitor of streptococcal deoxyribonuclease (Bernheimer, 1953). The absorption spectrum could not be distinguished from that of nucleic acid made from *TMV* by other methods.

In choosing optimum conditions for the fission, several considerations have to be borne in mind. $Sr(NO_3)_2$ solutions strong enough to dissociate *TMV* are good solvents for nucleic acid even when acid. The nucleic acid may be partly precipitated by the addition of ethanol, but the concentration of ethanol needed makes some of the $Sr(NO_3)_2$ crystallize out, so that a mixture of nucleic acid with the salt results. This difficulty can be avoided to a large extent if the action is not allowed to go so far as to precipitate all the protein, for then the residual soluble protein precipitates from the strong $Sr(NO_3)_2$ solution on addition of acid, and brings with it nearly all the nucleic acid that had been separated. But under these conditions the separation from the original protein coagulum is incomplete. It is necessary to choose, therefore, between incomplete fission with nearly complete recovery of the nucleic acid, and complete fission with difficult, and generally incomplete, recovery. The course of a representative experiment giving good recovery of nucleic acid is set out.

20 ml. of a 20 g./l. solution of *TMV* (2·1 mg. P) were added to 30 ml. of 2M-$Sr(NO_3)_2$, each previously adjusted to pH 8·5. The pH fell immediately and was restored to 8·5 by periodical addition of 0·1M-KOH; altogether, this required 1·0 ml. In 10 min. at 20° there was a heavy opalescence and no anisotropy of flow nor shimmer was detectable; in 20 min. a precipitate settled. After 70 min. its texture did not appear to change. The mixture was evacuated briefly so as to remove the air entangled in the coagulum; this air would have prevented clean separation on the centrifuge. The coagulum was centrifuged off and washed with 20 ml. of water. Acetic acid was added to the combined supernatants and the precipitate separating at pH 4 was centrifuged off and washed with 3 ml. of water. 2 ml. of conc. HNO_3 were added to the chilled fluids and the gummy precipitate of nucleic acid and a little protein was allowed to settle at 0° for a few hours; it was then centrifuged off and washed with 2 ml. of water. Most of the protein was in the first coagulum, and this contained 160 μg. P, the smaller precipitate of protein, separating at pH 4, contained 470 μg., the nucleic acid precipitate 1·3 mg., and the final fluid about 100 μg. P (this last estimation is uncertain because of the large amounts of Sr present). Nucleic acid was separated from the small protein precipitate thrown out at pH 4 by suspending it in 5 ml. of water at pH 8·5 and adding 0·5 ml. of saturated $(NH_4)_2SO_4$ solution also at pH 8·5. The protein precipitates along with very little of the nucleic acid; this was then precipitated by adding a few drops of conc. HCl and the precipitate was washed on the centrifuge.

The combined nucleic acid precipitates were carefully dissolved by adding KOH solution to pH 6 and then acetic acid to pH 4. After lying for some days at 0°, a small amount of protein was removed by centrifuging at 8000 rev./min. and the nucleic acid was again precipitated from the clear fluid at pH 1. This precipitate was washed, dissolved by adding KOH to pH 5, centrifuged clear if need be, and dried while frozen. The yield was 19 mg. of potassium nucleate, containing 8·5% P, i.e. 1·6 mg. P or 76% of that present in the original *TMV*. Some more nucleic acid can be separated from the protein precipitates by dissolving at pH 8·5 and throwing out most of the protein with $(NH_4)_2SO_4$ as already described; it can also be separated from the acid $Sr(NO_3)_2$ solution by the addition of ethanol.

The P content of precipitates separating from acid solution was determined by a method based on that of Kuttner & Lichtenstein (1932). Other precipitates and fluids contain Sr and these were evaporated to small volume in an oven at 100° with an excess of H_2SO_4. They were then taken up in water, centrifuged after standing for an hour, and the acid solution poured off from the $SrSO_4$ for analysis. Control experiments with known amounts of nucleic acid mixed with Sr salts showed that the recovery of P by this method was satisfactory.

Experiments with other nucleoproteins. Under the conditions used here, the other available nucleoproteins have not undergone fission in the same way as *TMV*. Thus, tomato bushy stunt virus gradually goes opalescent in $Sr(NO_3)_2$ solutions which are more concentrated than 1·2M and there is some precipitation, but there is little separation of nucleic acid from the protein. There is more separation from preparations of the Rothamsted strain of tobacco necrosis virus, but conditions have not been found that lead to such sharp separation as from *TMV*. With these two virus preparations it is necessary to dilute the reaction mixture to about 0·5M-$Sr(NO_3)_2$ before centrifuging, for stronger solutions keep some of the denatured protein dissolved. The normal nucleoprotein from young tobacco leaves (Pirie, 1950) is easily separated into protein and nucleic acid by treatment with alkali, and with trichloroacetic acid, under conditions that do not cause fission of *TMV* and the other viruses, but it is relatively resistant to $Sr(NO_3)_2$. The nucleoprotein is precipitated by low concentrations, but if a solution is added to 4 vol. of 2·4M-$Sr(NO_3)_2$, there is no precipitation immediately unless the mixture is diluted to about 0·5M. After a few minutes an opalescence develops, followed by flocculation of most of the protein. Even after 5 hr. at pH 8, however, the precipitate still contains one-third of the nucleic acid and much of the remainder pre-

cipitates along with the small amount of protein that precipitates with acid. The nucleic acid can be separated from this if the protein is dissolved in dilute alkali and then precipitated by the addition of 0·1 vol. of satd. $(NH_4)_2SO_4$ sol.; from the supernatant fluid the nucleic acid can be precipitated by adding acid. This method of preparation may have advantages in some circumstances, but it does not have the simplicity of the preparation from *TMV*.

Experiments with nucleic acid. In an attempt to bring about fission of the more resistant nucleoproteins, some tests were run at 37° and in them a protein coagulum free from nucleic acid resulted, but no nucleic acid could be isolated from the fluid. Commercial yeast nucleic acid and *TMV* nucleic acid made by the method already described were therefore incubated with 1·6 M-$Sr(NO_3)_2$. At intervals, samples were withdrawn and diluted with 2 vol. of water, and 10 N-HCl was added to a final concentration 0·1 N. The dilution is necessary because nucleic acid is soluble in acid $Sr(NO_3)_2$ solutions which are more concentrated than 0·7 M. After a few hours' incubation, precipitation with acid was impaired and there was no precipitation after 24 hr. at 37° and pH 8·5. There is, however, precipitation in these fluids—to an extent depending on the duration of incubation—on the addition of ethanol and almost all the P is precipitated by the addition of uranyl nitrate. It is clear, therefore, that this type of fission resembles fission with alkali in that the conditions have to be controlled or the nucleic acid will be destroyed as well as being liberated.

Experiments with other salts. This fission by $Sr(NO_3)_2$ was noticed during work on the inactivation of tomato bushy stunt virus by freezing in different environments (Bawden & Pirie, 1943*b*), and on the crystallization of the virus from various salt solutions (cf. Pirie, 1945). A thorough search for other salts with the same property has not been made, but a few related ones have been tested.

$SrCl_2$ is more soluble than the nitrate, but even in saturated solution it only causes partial precipitation of *TMV* in a paracrystalline state without splitting off the nucleic acid. $Ba(NO_3)_2$ is much less soluble, and a saturated solution makes *TMV* opalescent, but causes little precipitation and less fission. $Ca(NO_3)_2$, on the other hand, precipitates *TMV*. The suspension has a similar appearance to suspensions made by the action of the strontium salt, but the protein precipitate carries down with it most of the P. Various modifications in technique have been tried but a satisfactory yield of nucleic acid has not yet been got after treatment with $Ca(NO_3)_2$.

DISCUSSION

One point stands out clearly from these experiments as it did from earlier experiments (Bawden & Pirie, 1940*a*, *b*) with urea and some other agents—plant viruses differ greatly in the ease with which their nucleic acid can be separated from their protein. In some viruses the nucleic acid may not be connected at all, but held in a protein cage as Markham (1953) suggests with turnip yellow mosaic virus, though this suggestion does not seem to be really necessary (Pirie, 1953). It is conceivable that in other viruses the link is salt-like; the mutual precipitation of proteins and nucleic acids is well known. Greenstein (1944) has suggested that viruses are constructed in this way, but such a structure is hardly compatible with the behaviour of viruses such as *TMV* and tomato bushy stunt, which can be sedimented intact on the ultracentrifuge over a wide pH range and in environments of varied ionic composition. Although alike in this respect, these two viruses differ in the ease with which protein and nucleic acid can be separated. Thus, boiling, or exposure to such agents as strong acetic acid, urea, phenol, etc., is sufficient with *TMV*, but not with the bushy stunt virus, although some of these treatments cause a solution of it to coagulate. The same difference appears in the experiments described in this paper. Tobacco necrosis virus and normal leaf nucleoprotein are intermediate in their behaviour. Several explanations are possible. Some nucleoproteins may give a denatured protein with a physical structure that impedes the diffusing away of the nucleic acid, even although any chemical link between the two has been broken. This explanation would account for the lack of success that sometimes attends attempts to separate nucleic acid from *TMV* precipitates that have been made by adding insufficient $Sr(NO_3)_2$, but it has little else to recommend it. There may, on the other hand, be differences in the affinity of different denatured proteins for nucleic acid, or the link, in different viruses, may be of a chemically different nature. Such evidence as we have so far does not enable a choice to be made between these possibilities.

Among the possible mechanisms of this action, two extremes may be considered. The $Sr(NO_3)_2$ may break the linkage between protein and nucleic acid so that the two well-recognized parts of *TMV* fall apart; any changes there may be in the parts, denaturation of the protein for example, would then be secondary. The action, on the other hand, may be on one or other of the parts so that it is modified in such a way as to be incapable of maintaining the union between them. The observations made so far do not exclude either possibility. The properties of nucleic acids and their breakdown products are well known to be affected by salts; thus Tamm, Shapiro & Chargaff (1952) increased the diffusibility of degraded deoxyribonucleic acid from thymus by exposure to 0·2 M magnesium sulphate and Markham & Smith (1952) got similar results with several ribonucleic acid 'cores' in 2 M sodium chloride. Strontium nitrate, under rather more extreme conditions than those used to cause fission of *TMV*, makes ribonucleic acid from both yeast and *TMV* unprecipitable by hydrochloric acid, and it is reasonable to assume that it has some effect under the conditions used for fission, so that the first step may be a modification in the nucleic acid moiety of the virus. After separation the protein may well be more readily denatured than it was when combined. The protein that Sreenivasaya & Pirie (1938)

separated from *TMV* by treatment with sodium dodecyl sulphate was unstable, and other proteins also are more easily denatured after separation from their prosthetic groups. The presence of undegraded nucleic acid in solution with serum albumin has been found (Greenstein & Hoyer, 1950) to increase the thermal stability of the latter.

Many anomalies appear when the kinetics of the fission of *TMV* by alkali and the subsequent destruction of the liberated nucleic acid are studied. The results were partly systematized by Grégoire (1950), who found that 0·033 N sodium hydroxide acting for 4 min. at 18° split *TMV* into at least three products: protein that could be precipitated at pH 5·2 along with a little nucleic acid, nucleic acid which could be precipitated by hydrochloric acid along with a little protein, and a substance, which remained in the acid solution, that could catalyse the conversion of *TMV* nucleic acid into material not precipitated by hydrochloric acid. This action takes place in 0·033 N sodium hydroxide during a few hours and the advantage of using 0·5 N sodium hydroxide in the conventional methods for making nucleic acid is not that this strength of alkali is needed for the fission but that it is needed to suppress the secondary loss of acid precipitability. The high concentrations of strontium nitrate that are present when it is used to bring about fission would make it difficult to recognize any similar mechanism if it were playing a part here. But the possibility that *TMV* undergoes fission more readily than the other nucleoproteins because of the presence of other catalytic substance in it must be kept in mind in further work.

SUMMARY

1. Tobacco mosaic virus is split at room temperature into denatured protein and free nucleic acid by solutions of strontium nitrate if the concentration of the latter is greater than molar.

2. Other nucleoproteins are less easily split in this way and other related salts are not so efficient as strontium nitrate.

3. Nucleic acid is decomposed by more intense treatment.

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The Action of Some α-Amylases on Amylose

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The action of α-amylase is due to the fission of the α-1:4-glucosidic linkage in amylose and amylopectin. By confining the study to amylose and its fission products, complications arising from the presence of 1:6-glucosidic linkages are avoided. It is well known that the action of this enzyme is at first relatively rapid until the iodine colour disappears (achroic stage), this being usually attained in the case of amylose when about 20% of the linkages have been split. The mixture now consists of short-chain fragments including maltose and glucose (Myrbäck, 1948; Bernfeld, 1951). A much slower reaction, in some cases only one-hundredth as fast, overlaps and succeeds the first one. In this the short-chain fragments are successively split until only maltose and glucose or sometimes maltotriose remain. According to Myrbäck (1948), Bernfeld (1951), Meyer & Bernfeld (1941), Meyer & Gonon (1951), Alfin & Caldwell (1949) and Roberts & Whelan (1951) all except the terminal linkages in

the substrate molecule are attacked. So far as malt α-amylase is concerned Myrbäck & Sillen (1944) concluded that six linkages are less readily attacked than the remaining ones, at least one of these six being at one end of the chain in question. Thus when the chain has been reduced to one of seven glucosidic units, all of the linkages will now be less readily attacked than those of a large molecule and only the slow stage of the action can proceed. Myrbäck (1948) takes the view that, so far as malt α-amylase is concerned, glucose formation starts early in the reaction, and is split from large as well as small molecules. He cites results which support this opinion, although he states that much of the glucose ultimately formed is very slowly split from short-chain products. Meyer & Bernfeld (1941) and Meyer & Gonon (1951), on the other hand, consider that glucose owes its origin solely to the splitting of maltotriose, a reaction which can take place only after fission of amylose has proceeded far enough to produce some maltotriose. Admittedly some of this maltotriose fission must overlap the rapid (dextrinization) reaction, but the origin of glucose, in the view of these authors, is the fission of maltotriose. They further state that potato or maize amylose, acted upon by malt or pancreatic α-amylase, will yield essentially the same fission products at a given stage or percentage of linkages split. These fission products depend on stage of reaction rather than on the source of amylose or α-amylase. In contrast to these cases, Roberts & Whelan (1951) state that salivary amylase forms no glucose from amylose.

From the above summary it appears that (1) the stage at which formation of glucose can take place is uncertain and (2) in view of the overlapping of the two phases of the reaction it is uncertain what the first phase achieves, and whether even maltose is split from the original molecule. It becomes of importance to investigate the action of α-amylases on large molecules and to minimize the overlapping hydrolysis of short-chain fission products. Somogyi (1940), using starch as substrate, concluded that glucose is not directly split by α-amylase from starch or its high-molecular fission products, but is formed from lower-molecular (achroic) dextrins. The more dilute the reaction mixture the lower was the percentage of glucose in the reaction products at a given stage of hydrolysis or percentage of linkages split. He pictured these various fission products as competing for the enzyme, and dilution as enhancing the dissociation of the enzyme-substrate complexes, especially when the substrate molecules are small. Possibly diffusion plays a part here. The value of the Michaelis constant for starch components and the α-amylases of pancreas, malt and *Bacillus subtilis* appears to be less than 0·1% of substrate, whereas that of certain achroic fission products has been determined in this Department as upwards of 1·0% in the case of the malt enzyme.

If then amylose at, say 0·01–0·05% concentration, were hydrolysed by addition of these α-amylases we should maintain conditions for a relatively fast reaction for the breakdown of amylose and its higher molecular fission products whilst rendering that of the short-chain fission products extremely slow. Overlap of the former by the latter reaction would be negligible. One would expect the achroic stage (maximum chain length, 8 units) to be attained with a lower percentage of linkages split than is usual at this stage.

It is reported here (Tables 1–3) that this expectation was fulfilled, but that maltose and often glucose were already present, suggesting that the fission of these sugars had already taken place in the very dilute reaction mixture prior to the achroic stage.

To ascertain whether these fissions occurred at the reducing or non-reducing end, similar experiments were performed with amylopectin, all the end groups of which at the outset of the reaction would be non-reducing. Amylopectin (from waxy maize) contains one non-reducing end group/20 glucose units (Brown, Halsall, Hirst & Jones, 1948). At a certain stage of α-amylolysis amylose would contain the same proportion of non-reducing end groups as unhydrolysed amylopectin, but, in addition, an equal number of reducing ends, in this respect differing from amylopectin. The next phase of α-amylolysis of these two products (Tables 4 and 5) revealed differences. The appearance of, for example, maltose in the fission products of amylose and its non-appearance in the early products of amylopectin was attributed to an attack by the α-amylases on the second linkage from the reducing end of the chain and to their failure to split the second linkage from the non-reducing end. In the case of the α-amylases of malt and *B. subtilis*, other sugars such as maltotriose were absent from the early amylopectin fission products, but were present when salivary amylase was used.

In view of the need to detect relatively small amounts of these sugars in the reaction products and the need to work with very dilute reaction mixtures, paper chromatography was used, by means of which a few micrograms can be detected. In cases where sufficient quantities of the individual sugars were formed their presence was confirmed by other methods such as chromatographic separation on a charcoal/Celite column (Whistler & Durso, 1950; Bailey, Whelan & Peat, 1950) or measurement of optical rotation and reducing power. It was also considered desirable to confirm the nature of the final fission products of the α-amylases of malt and *B. subtilis* (Tables 8 and 9) by the latter methods since, with the exception of Roberts & Whelan (1951), who used a charcoal/Celite column in their

work on salivary amylase, the workers mentioned above used fermentation methods to determine glucose and maltose. There are objections to such methods, e.g. errors in the determination of small proportions of glucose and failure to distinguish between maltose and maltotriose.

The actions of the α-amylases of malt, *B. subtilis*, saliva and *Aspergillus oryzae* on certain amylose fission products (Tables 6 and 7) revealed minor differences from their actions on amylose itself, and the results as a whole enabled a picture of the pattern of these actions to be formed. The *A. oryzae* enzyme, however, was so contaminated with maltase and, apparently, a group-transferring enzyme, that the results are reported without making specific deductions.

EXPERIMENTAL

Preparation of substrates. Amylose was prepared from potato starch by the method of Schoch (1942), and recrystallized and stored as described by Hopkins & Jelinek (1948). We are indebted to Dr Schoch for the gift of waxy maize amylopectin.

The hexasaccharide (maltohexaose) and other achroic fission products of amylose were prepared by hydrolysis of the latter with *B. subtilis* α-amylase until colourless with iodine, boiling, and then concentration and chromatographic separation by the method of Whistler & Durso (1950), which has been applied to similar products by Bailey *et al.* (1950). The hydrolysate was passed through a charcoal/Celite column (170 × 34 mm.) and the constituents were eluted with successive aqueous solutions of ethanol of increasing concentration:

Constituent eluted	Concentration of aqueous ethanol (%)	Volume used (ml.)
Glucose	0	800
Maltose	5	900
Trisaccharide	15	500
Tetrasaccharide	20	200
Pentasaccharide	25	200
Hexasaccharide	30	350

The volumes are average values. The elution of an individual constituent was carried out until no further material came off the column. The concentrated eluate was tested by chromatography and by optical rotation.

Glucose, maltose and maltotriose were effectively separated by a single application of this procedure. The products used as substrates in Tables 6 and 7, precipitated with acetone and dried *in vacuo*, had the following properties: hexasaccharide, $[\alpha]_D$ (equilibrium in water) +176°, R_m 33, chain length (periodate) 6·1; maltotriose, $[\alpha]_D$ (equilibrium in water) +157°, R_m 64, chain length (periodate) 3·0.

In the preparation of amylose dextrin, α-amylolytic action was stopped when the iodine reaction was quite red; the dextrin was precipitated with acetone and dialysed for 2 days.

α-Amylases. We are indebted to Dr Schwimmer for the gift of a specimen of crystalline malt α-amylase, prepared by the method of Schwimmer & Balls (1949). Malt α-amylase was also prepared by the method of Schwimmer & Balls (1948). We are also indebted for commercial and purified preparations of bacterial amylases, prepared from cultures of *B. subtilis*, to Wallerstein Laboratories, New York, and to Norman Evans and Rais Ltd., who also supplied a fungal amylase 'amylozyme' (*A. oryzae*). Saliva, in which little or no maltase activity could be detected, was often used directly after having been diluted and centrifuged. However, the method of purification described by Meyer, Fischer, Staub & Bernfeld (1948) was also employed.

Determinations. R_m was determined by the method of Blom & Rosted (1947). This quantity is the reducing power calculated as maltose and expressed as percentage of the maltose equivalent of carbohydrate present. The value of R_m is twice the percentage of glucosidic linkages (of all kinds) split.

Periodate oxidations were performed by the method of Potter & Hassid (1948).

Absorption values (A.V.) were measured by running 1 ml. or other suitable volume into 50 ml. solution containing 10 mg. I_2 and 100 mg. KI, diluting to 100 ml., and reading in a Spekker absorptiometer, after it had stood for 1 hr. at 18°. Late readings, when hydrolysis was well advanced, were made on larger volumes of reaction mixture but the results were calculated to that volume corresponding to the initial withdrawal. During α-amylolysis the values of A.V. at all wavelengths when plotted against R_m fell rapidly at first, but later asymptotically approached zero. The fall is slower the shorter the wavelength. The achroic point is therefore vague, because R_m increases appreciably, while A.V. (at 470–490 mμ.) approaches zero.

Paper-partition chromatography was performed essentially as described by Partridge (1948). Volumes (5–20 μl.) of hydrolysate or control sugars were applied with a graduated capillary pipette at intervals along a line 10 cm. from the top of the paper (Whatman no. 1) and dried. A mixture of 10 ml. glacial acetic acid and 90 ml. of 75% (v/v) aqueous *iso*propanol as solvent gave the best movement and separation. Irrigation was at 26° for 18 hr. in which time the solvent had moved 25–30 cm. Development was by spraying with aniline hydrogen phthalate (Partridge, 1949) and heating to 100° for 5–15 min. Dextrins of 3–6 glucose units length required a longer heating time. The bacterial enzymes gave dark patches usually just above the spots corresponding to trisaccharide. It should be clearly understood that the absence of a spot does not necessarily mean the complete absence of the corresponding sugar, but that the quantity present was less than a certain amount. This, in the standard development procedure used, was about 2, 10 and 20 μg. for glucose, maltose and maltotriose, respectively.

It must also be made clear that, while the depth of a spot is indicated in the tables by a suitable number of '+' units, these numbers only afford a satisfactory basis for comparison on any one chromatogram series when they refer to the same sugar. Even then, when a certain depth of colour is exceeded, further increase becomes difficult to detect. In estimating the relative quantities of different sugars greater error is possible since, the longer the saccharide chain, the less the depth of colour. Thus whilst 20 μg. of sugar gave a pronounced spot in the case of glucose, with maltose it was much fainter and with maltotriose just detectable. However, bearing these points in mind, and with experience, it was quite possible to form rough estimates of the relative quantities of different sugars.

Reaction mixtures. Reaction mixtures were set up as stated in the tables, often without added buffers if paper chromatographic methods were to be employed. Antiseptics, both toluene and nitrobenzene, were always added if the reaction was to last for more than a few hours. Portions withdrawn at suitable intervals were boiled to stop enzymic action, concentrated as necessary, and portions of the concentrate used for the determinations required. Rapid arrest of enzyme action, when desired, was achieved by the addition of one or two drops of acetic acid and raising to boiling point as quickly as possible.

RESULTS

Action of α-amylases on amylose

Achroic point of amylose at medium and low concentrations. Table 1 shows the R_m values. The dilute (0·022 %) solutions were achroic at R_m 26–27, equivalent to a mean chain length of 7–8. Further fission of some of these chains, which took place in the 0·44 % reaction mixtures, was minimized by the use of very dilute solutions.

Table 1. *Achroic point of amylose hydrolysed at medium and low concentrations*

(Three reaction mixtures containing 0·44 % amylose were divided immediately after addition of the α-amylase. One half was at once diluted to 20 vol., and the reaction allowed to proceed in parallel.)

	R_m value at the achroic stage	
Enzyme	0·44 % reaction mixture	0·022 % reaction mixture
Malt (stock)	41·5	26·4
Malt (crystalline)	37	26·1
Bacterial	37·5	27·6

Hydrolysis of amylose at various concentrations by malt α-amylase. As in Table 1 the approximate achroic point was reached in dilute solution (0·025 %) at a much lower R_m value than at higher concentrations. This is indicated by the results shown in Table 2.

The chromatograms indicated large amounts of dextrins in the range of 6–8 units chain length, but much smaller amounts (often traces) just below 6 units chain length. Only in the case of 0·025 % concentration at R_m 22·8 and 30·2 was the glucose so faint as to be only just recognizable (in this case less than 0·2 % of the carbohydrate present).

Maltose and maltotriose were present in appreciable quantities even at R_m 22·8.

Hydrolysis of amylose at low concentration by bacterial amylase. Separate reaction mixtures were set up and were stopped at the stages indicated in Table 3.

Chromatograms were carried out on concentrates sufficient to detect as little glucose as 0·1 % of total solids. The chromatograms from *C* indicated not more than 0·5 % glucose and *D* more than 1 %. Both maltose and maltotriose had appeared much earlier (at R_m 7·8) and increased with R_m as was to be expected.

No glucose was seen on the chromatogram of 0·041 % amylose at R_m 18, which was run on a concentrate such that glucose equivalent to 0·04 % of the carbohydrate present would have been detected.

In Tables 4–7 crystalline malt and Wallerstein bacterial amylases were used, the latter giving chromatograms comparatively free from foreign patches.

Table 2. *Hydrolysis of various concentrations of amylose by crystalline malt α-amylase*

(Temperature, 33°. Maltose and maltotriose present on all chromatograms.)

Amylose concn. in reaction mixture (%)	R_m value	Loss in A.V. (%) at 680 mμ.	600 mμ.	490 mμ.	470 mμ.	Chromatographic detection of glucose
0·46	40	99	99	93	91	+ +
0·25	38	100	99	93	91	+ +
0·077	33	99	99	94	93	+ +
0·025	22·8	99	98	80	75	Trace
0·025	30·2	–	–	–	98	Trace
0·025	35·7	100	100	99	99	+

Table 3. *Action of bacterial α-amylase on amylose*

(*A–E* are five separate experiments. *A–D*, amylase, 0·84 %, pH 6; reactions stopped at stages shown. *E*, amylose 0·041 %. The reaction ceased at R_m 18. The reaction mixtures were unbuffered and incubated at 33°. Maltose and maltotriose present on all chromatograms.)

	R_m	Loss in A.V. (%) at 680 mμ.	490 mμ.	Chromatographic detection of glucose
A	7·8	39	26	–
B	9·8	74	31	–
C	30·6	99	85	+
D	47·6	100	100	+
E	18	–	–	–

Fission products of dilute amylose near the achroic point. Table 4 shows the chromatograms. The control standards correspond to yields of 1·6 and 0·16 % of the amylose, respectively.

In the case of malt and bacterial α-amylases, dextrins of 6–7 units chain length, maltotriose and maltose predominated, but only slight traces of tetra- and penta-saccharides and of glucose were present. In the case of salivary and fungal amylases little material of 6–8 units chain length remained. Whereas in the case of malt and salivary amylases maltose appeared in greater yield than maltotriose, in that of bacterial and fungal enzymes the reverse was the case. Maltase, however, may be responsible for this in the case of the fungal enzyme.

Separation of the products in a charcoal/Celite column confirmed the presence of these fragments and, in particular, the presence of dextrins of both 6 and 7 units chain length in the malt and bacterial amylolytic products. The first fractionation gave a mixture of 6 and 7 units chain length. This, partly hydrolysed by crystalline β-amylase at pH 3·6, yielded maltose plus tetra- and penta-saccharide as shown by paper chromatograms, still later products being maltose and maltotriose.

Table 4. *Fission products of* 0·025 % *amylose near the achroic point*

(0·025 % amylose and α-amylase were incubated at 33° and the reaction was stopped as nearly as possible at the achroic point. The chain lengths of the fission products were determined by control chromatogram. The symbols '+', '2+', etc. give rough estimates of the relative quantities of the different sugars as shown on the chromatograms. For further data see Experimental section.)

	α-Amylase				Standards (as % of substrate)	
Chain length	Malt	Bacterial	Salivary	Fungal	1·6	0·16
8	2+	+	+	+	–	–
7–6	3+	4+	–	+	–	–
5	–	+	–	3+	–	–
4	–	–	2+	2+	–	–
3	+	5+	5+	4+	–	–
2	3+	2+	8+	+	Very faint	–
1	Very faint	Very faint	–	8+	4+	+
	R_m 30	40	35	46		
Loss in A.V. (470 mμ.) (%)	98	100	89	99		

Table 5. *Early fission products of* 0·027 % *amylopectin*

(Enzyme and substrate were incubated at 33°; reactions were stopped at an early stage. The chain lengths of the fission products were determined by control chromatogram. The symbols '+', '2+', etc. give rough estimates of the relative quantities of the different sugars as shown on the chromatograms. For further data see Experimental section.)

	α-Amylase					Standards (as % of substrate)	
			Salivary				
Chain length	Malt	Bacterial	(1)	(2)	Fungal	1·0	0·1
Undefined but large	10+	6+	+	2+	6+	–	–
6	+	+	+	4+	8+	–	–
5	–	–	+	4+	8+	–	–
4	–	–	2+	6+	8+	–	–
3	–	–	4+	12+	8+	–	–
2	–	–	–	+	3+	4+	–
1	–	–	–	?+	12+	16+	2+
Loss in A.V. (470 mμ.) (%)	30	11	7	11	57		

Early fission products of α-amylase on dilute amylopectin

Amylopectin (0·027 %) and α-amylase were incubated at 33° under conditions similar to those in Table 4 except that the reactions were stopped at a much earlier stage for reasons already discussed. Table 5 shows the chromatograms. The control standards correspond to 1 and 0·1 % of amylopectin, respectively.

Very little material of chain length less than 6 units was formed by the malt and bacterial enzymes, but considerable amounts of tri-, tetra-, penta- and hexa-saccharide were formed by the salivary and fungal enzymes. The latter as usual also formed much glucose but it had carried the reaction to a 57 % fall in A.V. One important point is that maltotriose made its appearance at a very early stage of fission by salivary amylase, another is the comparative absence of maltose from the products of malt and salivary amylases.

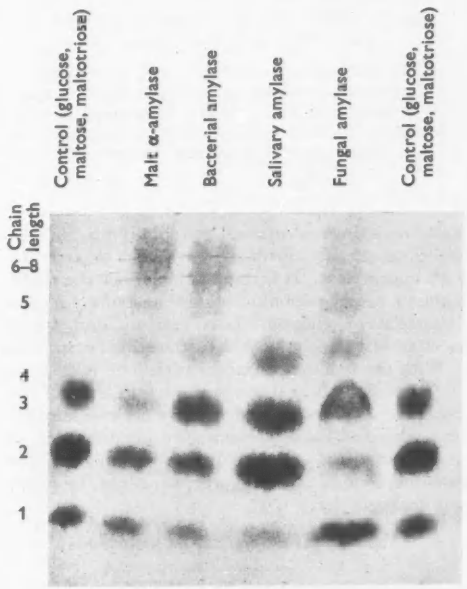

Fig. 1. Fission products of amylose dextrin of mean chain length approximately 20 units, near the achroic point.

	% A.V. lost		Glucose (%) from chromatogram
	680 mμ.	490 mμ.	
Malt (crystalline)	97	88	0·5
Bacterial	98	94	0·05
Salivary	97	92	0·01
Fungal	95	87	5·0

Fission products of amylose dextrin of mean chain length approximately 20 units

Dextrin (0·82 %) and α-amylase were incubated at 33° under conditions similar to those in Table 4. Chromatograms and absorption values were read at intervals on samples withdrawn without concentration. Whereas using salivary amylase under these conditions maltose, maltotriose and tetraose had appeared before the A.V. (490 mμ.) had fallen by 40 %, when using malt amylase these fission products had not appeared by 80% fall in A.V. Using the bacterial enzyme, maltotriose had appeared at 60 % fall in A.V. However when samples taken near the achroic stage were concentrated to one-tenth volume, the chromatograms appeared as in Fig. 1.

These indicate that the main fission products at the achroic stage were, in the case of salivary amylase, maltose, maltotriose and tetraose, and in the case of the malt and bacterial amylases, dextrins of 6–7 units chain length. All of these were also the first products to appear on the chromatograms run on the earlier withdrawals. It is concluded that the malt and bacterial amylases are better able to split linkages 6 or more units from the end of a chain than within this distance. The presence of some fission products of less than 6 units chain length, however, in these and the amylose reaction mixtures of Table 4, and their absence from the amylopectin early hydrolytic products suggest that they are split by these two enzymes from the reducing ends of chains. Salivary amylase however split linkages nearer to the ends much more readily than the other enzymes, but failed to split the second link from the non-reducing end or to split glucose from either end.

The fungal enzyme yielded products intermediate between those of the bacterial and salivary amylases, but with additional glucose, presumably due to maltase activity.

Fission products of linear hexasaccharide

The results are shown in Table 6.

Malt α-amylase. The first fission products were maltose plus tetrasaccharide, with smaller amounts of glucose plus pentasaccharide, the glucose appearing too soon for it to be a product of fission of trisaccharide. The latter however appeared soon. Tetrasaccharide did not diminish until hexasaccharide had disappeared. The final products were glucose, maltose and maltotriose.

It was clear that the fission products most readily formed were maltose and maltotetraose. The maltotriose spots were not only lighter but represent both the product split off and the residue in the reaction hexasaccharide→2 trisaccharide.

Bacterial α-amylase. Much less maltose but more glucose were formed than with malt α-amylase, which it otherwise resembled. With the glucose,

Table 6. *Progress of action of α-amylase on linear hexasaccharide*

(Hexasaccharide (0·78%) and α-amylase were incubated at 33°. A maltase control was run under strictly parallel conditions in the salivary amylase experiment. Chain length of saccharide and fission product as indicated by control chromatogram. The first column in the table represents a chromatogram run on the substrate alone. Times refer, in the case of salivary amylase, to actions on both substrates. The symbols '+', '2+', etc. give rough estimates of the relative quantities of the different sugars as shown on the chromatograms. For further data see Experimental section.)

Chain length	Malt α-amylase (crystalline)									Bacterial α-amylase (Wallerstein)				
6	4+	4+	4+	3+	3+	2+	+	–	–	3+	2+	+	–	–
5	–	–	–	+	+	+	–	–	–	+	2+	3+	3+	+
4	–	–	+	+	2+	2+	3+	2+	+	+	+	+	+	+
3	–	–	–	–	+	+	2+	2+	+	–	+	2+	2+	3+
2	–	+	+	2+	2+	3+	3+	4+	4+	+	+	2+	2+	3+
1	–	+	+	+	+	2+	2+	2+	2+	+	2+	3+	3+	4+
R_m	33	40	–	–	46	62	77	87	87	34	44	54	75	85
Time (hr.)		0·2	–	–	–	1	2	3	–	–	0·25	0·5	1	2

Chain length	Salivary α-amylase							Fungal α-amylase (*Aspergillus*)				
6	4+	+	+	–	–	–	–	3+	2+	–	–	–
5	–	–	–	–	–	–	–	–	–	–	–	–
4	–	+	+	–	–	–	–	–	–	+	+	–
3	–	+	+	+	3+	3+	2+	+	2+	2+	3+	4+
2	–	+	2+	3+	4+	6+	6+	–	–	+	2+	3+
1	–	–	–	–	–	+	2+	–	–	+	3+	4+
R_m	33	–	35	39	–	75	–	–	34	38	77	–
Time (min.)	–	2	6	25	120	180	300	Maltose control for salivary amylase				
2	2+				2+		2+					
1	–				–		–					

pentasaccharide appeared and increased for a time. The glucose was therefore split directly from the hexasaccharide. This enzyme readily split the end linkage of the hexasaccharide, in contrast to its action on longer chains (Table 4 and Fig. 1). The final products were glucose, maltose and maltotriose.

Salivary α-amylase. This enzyme split no glucose directly from a hexasaccharide but mainly split it into maltose and tetrasaccharide. Maltotriose also was formed from the outset, but in view of the consistently deeper colour of the maltose spots and having regard to the fact that two molecules of maltotriose are formed by one fission, it was concluded that the second linkage from one end of the maltohexaose was more readily attacked than the third. In view of the results of Table 5 this linkage cannot be the second from the non-reducing end. The very late, small yield of glucose seems to be accounted for by the action of the enzyme on maltotriose, confirmed by a later direct experiment, especially as the maltose was simultaneously increasing and the maltotriose decreasing. Maltase activity was negligible and was not detected in this experiment, but may be responsible for some of the glucose.

Fungal α-amylase. Fungal α-amylase resembled the salivary enzyme, but had a more marked action on the resulting maltotriose, and split it into maltose and glucose, as confirmed by a direct experiment with maltotriose. It is doubtful whether the slight maltase activity could wholly account for the formation of glucose. Less maltose and tetrasaccharide were formed than by the salivary enzyme.

With the reaction mixtures used here (except in the case of the fungal enzyme), the presence of the fission products was confirmed by their separation on a charcoal/Celite column; the identification of glucose, maltose and maltotriose was made, when possible, by optical activity or, when not, by paper chromatography.

Action of the α-amylases on maltotriose and maltose

All of the four enzymes in this experiment (Table 7) were able to split maltotriose: the feeble maltase activity of the salivary enzyme and stronger one of *Aspergillus* were quite inadequate to account for the rate of fission of maltotriose. Many samples of human saliva from different sources were tested for maltase against control runs with maltose alone. By this rigorous test a slight and variable maltase activity was always found, except in the instance recorded in Table 6.

Table 7. *Action of α-amylases on maltotriose and maltose*

(Maltotriose (0·4%) or maltose (0·26%) incubated with α-amylase at 33°. Chain length of substrate or fission product as indicated by control chromatogram. The symbols '+', '2+', etc. give rough estimates of the relative quantities of the different sugars as shown on the chromatograms. For further data see Experimental section.)

	Substrate						Substrate			
	Maltose			Maltotriose			Maltose		Maltotriose	
	Malt α-amylase (crystalline)						Bacterial α-amylase (Wallerstein)			
Chain length										
3	–	–	–	4+	3+	2+	–	–	4+	3+
2	4+	4+	4+	–	+	2+	4+	3+	–	+
1	–	–	–	–	+	+	–	–	–	+
	Salivary α-amylase						Fungal α-amylase (*Aspergillus*)			
3	–	–	–	4+	2+	–	–	–	+	–
2	4+	4+	4+	–	2+	4+	4+	2+	2+	+
1	–	–	+	–	2+	4+	–	2+	3+	4+
Time (hr.)	0	4	22	0	4	22	4	22	4	22

Table 8. *Fission products of action of malt α-amylase on amylose to* 97 R_m

(Amylose (0·72%) and malt α-amylase were allowed to react at pH 5·8 and 60° in the presence of antiseptics with subsequent additions of enzyme for 6 days. After filtration and concentration, the syrup was precipitated with acetone.)

	Percentage of total hydrolytic products		
	Precipitated with acetone to 67% (v/v)	Filtrate from acetone	Total
Glucose	Trace	5	5
Maltose	25	55	80
Maltotriose (including a trace of tetrasaccharide not determined separately)	15	Trace	15
Chromatograms			
Glucose	Slight trace	+	
Maltose	+	+	
Maltotriose	+	Slight trace	
Tetrasaccharide	Trace	—	

Prolonged action of malt α-amylase on amylose

Amylose (0·72%) and malt α-amylase were allowed to react at pH 5·8 and 60° in the presence of antiseptics with subsequent additions of enzyme until, after 6 days, reducing power corresponded to R_m 97·0. After separation of the small amount of flocculum and concentration to a syrup, precipitation with 2 vol. of hot acetone was followed by reprecipitation of a solution of the precipitate under the same conditions. The clear supernatant portions and washings were collected, freed from acetone and analysed, while the reprecipitated fraction was similarly treated. Analysis by paper chromatography indicated which constituents (two in each case) were present in the respective fractions. Calculation of these amounts from specific rotation and reducing power was made by the usual methods; the results are given in Table 8. A little of the reaction mixture at R_m 97·0 was fractionated on a charcoal/Celite column. Glucose, maltose and maltotriose were recovered in quantity, and identified by specific rotation, thus further confirming the results of paper chromatography.

The acetone precipitate (11·8 mg. maltotriose and 19·7 mg. maltose/ml.) was treated with such a quantity of malt α-amylase as would be equivalent (per unit of carbohydrate present) to 4 times the total used in the original hydrolysis. After some days the reaction appeared to have ceased. Analysis now gave (per ml.): 4·2 mg. maltotriose, 24·3 mg. maltose, 2·7 mg. glucose. Action of the amylase on the maltotriose had been exceedingly slow.

In a control experiment, maltose and enzyme were incubated under the same conditions. No maltase activity could be detected by change in either α_D or reducing power, but a correction for the small optical activity of the enzyme preparation was obtained from the readings.

Prolonged action of bacterial α-amylase on amylose

The previous experiment was repeated at pH 5·8 and 45° using bacterial instead of malt α-amylase. At the achroic stage, R_m 37, a portion of the reaction mixture was concentrated and fractionated on a charcoal/Celite column. Glucose (trace), maltose and maltotriose were separated and identified, and higher saccharides, less completely separated, were also present.

Table 9. *Fission products of action of bacterial α-amylase on amylose to completion*

(Amylose (0·72 %) and bacterial α-amylase were allowed to react at pH 5·8 and 45° in the presence of antiseptics with subsequent additions of enzyme for 4 days. At R_m 74 a portion was precipitated with acetone (67 %, v/v), the precipitate redissolved in water and treated with more enzyme for 6 days.)

	Percentage of total hydrolytic products		
	Filtrate separated by precipitation with acetone at R_m 74	Remainder (formed in complete hydrolysis) of precipitated portion	Total
Glucose	2	6·4	8·4
Maltose	18	73·6	91·6
Maltotriose	—	Trace	Trace

	Chromatograms (taken from whole reaction mixture)	
	At R_m 82	At R_m 90
Glucose	+	+
Maltose	+	+
Maltotriose	+	+
Tetrasaccharide	+	+ (very faint)

The reaction was continued as far as R_m 74 (4 days) when analysis of a portion by the same procedure as in Table 8 revealed the presence of 2 % of glucose in the reaction products at this stage. The portion precipitated by acetone amounted to about 80 % of the whole. This was not analysed at this stage but was redissolved in water, treated with more enzyme until action seemed to be complete (10 days, R_m 107) and analysed as in Table 8. The results are shown in Table 9.

The final products were glucose, maltose and a trace (undetermined) of maltotriose.

Portions of the total reaction mixture at the stages R_m 82 and 90 gave paper chromatograms as described in Table 9.

A maltase control was performed but no maltase activity could be detected.

DISCUSSION

Action of α-amylases on terminal and adjacent linkages

It will be convenient to refer to the rapid phase of the reaction which is terminated at about the achroic stage as 'dextrinization', and the subsequent fission of the achroic products as 'secondary fission'. It is concluded from the results of Tables 1 and 2 that the α-amylases of malt and *B. subtilis* split the amylose molecule in very dilute solution into chains of about 8 or 7 units length, i.e. short enough to be achroic, but very little further since R_m was 27, equivalent to a mean chain length of 7·4. It is true that a relatively small proportion of dextrins giving a faint-brown colour with iodine was still present, and to minimize secondary fission the reaction was arrested before these had quite disappeared. Although little or no fission of short chains could have occurred, maltose, maltotriose and slight traces of glucose were already present and had presumably been split off from longer chains. There were indications that less glucose is split from amylose by the bacterial than by the malt enzyme (Tables 2–4, Fig. 1) and none by salivary amylase, but all split off maltose.

To study the effects of the amylases on the outermost chains of amylopectin it was necessary to employ dilute solutions and stop the reactions early to prevent or minimize further action on the fission products (Table 5). The corresponding hydrolyses of amylose (Table 4) had to be carried further to be comparable, since the attainment of R_m 10 is necessary as a preliminary to bring the concentration of non-reducing end groups to the same value as that in the unhydrolysed amylopectin. Any marked differences between the low-molecular fission products in the two experiments in the next stages will be attributable to the action of the amylase on the linkages near the reducing ends. Such differences are most marked in the case of salivary amylase, which liberated much maltose from amylose and very little from amylopectin. The same effect was observed with malt α-amylase. The bacterial enzyme split maltotriose the most readily from early fission products of amylose but not from amylopectin. The conclusion is drawn that the salivary, malt and bacterial amylases readily attack the second, second and third linkages, respectively, from the reducing end. Linkages further from the end are also attacked (Tables 2 and 3, Fig. 1), unless they are within the proscribed distance from the non-reducing end, as is discussed below.

Features common to the hydrolysis of both substrates and, in particular, observed in the early stages with amylopectin, can well be attributed to enzyme action on linkages within a few units of the non-reducing end. Such a feature is the formation of tri- and tetra-saccharide by salivary amylase. It is noteworthy that malt and bacterial amylases split off from amylopectin only negligible traces of saccharides of less than 6 units length. It is clear, however, that the salivary enzyme can split the third linkage from the non-reducing end and other linkages further on, unless within the proscribed distance of the reducing end. The second linkage from the non-reducing end is split very much more slowly as in the fission of maltotetraose. Fission products containing α-1:6 linkages could not be smaller than 5 glucose units (Whelan & Roberts, 1952) and would not be represented on the chromatograms in the positions occupied by tri- and tetra-

saccharide. Even in the pentasaccharide position a branched compound is unlikely in the very early reaction products. Furthermore, while the proximity of α-1:6-linkages may influence the susceptibility of certain α-1:4-linkages to fission, the latter will be those which are furthest from the non-reducing ends. Failure of salivary amylase to split the second, and malt and bacterial amylases the second to fifth linkages from the non-reducing ends in amylopectin cannot be ascribed to the presence of 1:6-linkages situated several units along the chain.

The linear dextrin used in Fig. 1 resembles amylopectin in some respects such as percentage of non-reducing end groups. Its fission products with the respective enzymes support the conclusions drawn above.

In contrast to the salivary enzyme, the malt and bacterial amylases in Table 5 split off only negligible traces of saccharides of less than 6 units length. It is concluded that they are only able to attack the first five linkages from the non-reducing end very slightly or slowly. That these enzymes split the second and third links from the non-reducing end with difficulty is confirmed by the slow disappearance of maltotetraose and triose (Table 6). While resembling one another in this respect, malt and bacterial amylases differ in their attack on the linkages near the reducing end, malt readily attacking the second but bacterial amylase the third linkage (Table 4, Fig. 1). Similarly, malt α-amylase splits the first with much less ease than the second, while the bacterial enzyme splits the second linkage much less readily than the third, and the first even less easily. This has the effect that whereas five linkages at the non-reducing end and one at the reducing end, i.e. in all six linkages, are relatively immune to fission by malt α-amylase, five and two, or seven linkages, are correspondingly immune to the bacterial α-amylase. Thus, the bacterial amylase enters the slow, purely saccharification phase, 'secondary fission', at an earlier stage than is the case with the malt enzyme, i.e. when the substrate has been split to chains of seven linkages (chain length 8) as against six (chain length 7). (Cf. Hopkins & Kulka (1942) who used an enzyme from the same source.) But the minimum length of chain which results in any quantity from the dextrinization phase by these enzymes is 6, for the last fission by malt has usually removed not less than two and bacterial amylase not less than three units.

Although the bacterial amylase splits glucose from chains of longer lengths less readily than does malt α-amylase, yet relatively more glucose appears to be formed in its action on the hexasaccharide (Table 6). This, however, is because the bacterial enzyme is in this case unable to exercise freely its function of splitting off trisaccharide owing to the proximity to the non-reducing end of the linkage involved. The result is that relatively less maltotriose and maltose are formed and more of the enzyme action is forced on to the linkage next to the reducing end, yielding glucose. To a certain extent the same influence is seen in the fission of the hexasaccharide by malt α-amylase. This provides an additional reason why glucose formation becomes noticeable in the advanced stages of amylose hydrolysis. As the substrate chains become shorter, glucose constitutes an increasing proportion of the fission products. Glucose makes an earlier appearance in the fission of amylopectin or starch than of amylose (Myrbäck, 1948; Alfin & Caldwell, 1949). This also is due to the earlier appearance of short-chain fission products.

The possibility that β-glucosidic residues in amylose (Peat, Thomas & Whelan, 1952) may give rise to traces of glucose has been considered. Such residues could not be present in sufficient quantity to account for the yields of glucose liberated, especially those from the short-chain substrates, as in Fig. 1 and Table 6. β-Glucosidase activity was not found in the malt α-amylase, which yielded more glucose from amylose than did the bacterial enzyme. The fungal enzyme was admittedly far from pure, whilst the salivary enzyme split no detectable quantity of glucose except from the hexasaccharide and maltotriose.

Salivary amylase differs from the malt and bacterial enzymes in the readiness with which it splits the third linkage from the non-reducing end and the two linkages beyond. It also differs from malt α-amylase in its incapacity to split the first linkage from the reducing end, but resembles it in that it readily attacks the second linkage from the reducing end. With salivary amylase, chains of 5 units or more can be readily split at the second linkage from the reducing end, but a chain of 4 units will be only slowly split at its middle linkage (cf. Fig. 1).

The calculation of Meyer & Gonon (1951) indicates that salivary amylase should split amylose finally yielding 2·35 molecules of maltose to each one of maltotriose, or approximately seven to three (Roberts & Whelan, 1951). If, before the end of the reaction, some of the seven molecules of maltose were still present as tetrasaccharide undergoing slow fission, we could well imagine the reaction as entering on the slow secondary phase with two tetrasaccharide and three maltose to every three maltotriose molecules. This would correspond to R_m 70. The relative quantities of the oligosaccharides in the imaginary case correspond quite well with those which can be deduced from the chromatograms (Table 4, Fig. 1). It is to be expected that when amylopectin is the substrate, the increased ability to split off maltotriose in the early stages

would influence the final yield of this sugar, and the ratio would become less than 2·35:1, as was actually found by Whelan & Roberts (1952).

No glucose was ever detected in actions of salivary amylase on amylose, and only in those cases where the reaction conditions permitted the prolonged exposure of maltotriose to the action of the enzyme was any glucose formed. In Table 7 there was significantly more action on maltotriose than on maltose. The faint maltase activity could scarcely account for the complete fission of the maltotriose into maltose and glucose. The further fission of the resulting maltose was not accomplished. It is, however, possible that for some reason, the maltase acts on maltotriose much more rapidly than on maltose.

Here again we see the influence of the non-reducing end. When no available linkage remains save the one next to the reducing end, the salivary amylase is forced to act on this linkage. In maltotriose, the proximity of the non-reducing end is equivalent in its effect on salivary amylase to its effect on the bacterial amylase when the hexasaccharide is attacked, as discussed above. If pancreatic amylase resembles our salivary amylase in this respect, the conclusion of Meyer & Gonon (1951) that glucose is formed solely by fission of maltotriose would be justified so far as this enzyme is concerned.

Specific action of α-amylase. General picture

Before proceeding further, it seems desirable to form a general picture of the specific union of α-amylases with their substrates. For simplicity, no attempt has been made to depict the linear glucosidic chain as helical in form.

Let us represent the α-1:4-glucosidic chain by arrows joined as in Fig. 2, where each arrow represents a glucosidic residue, and the arrow head the reducing carbon atom. The α-amylase may be regarded as having a specific zone *A–B* enabling it to unite with the substrate molecule along, say (as in the case of bacterial amylase) 9 glucosidic units of its length, as shown in the diagram at *C*. Fission follows at the point indicated, the 'tension' necessary to effect this being easily brought to bear if the full number of glucosidic units possible had been united to the enzyme. When the chain overlaps the specific zone of the enzyme (as in *C*) this will always happen, union will be easy, affinity constant high and fission rapid.

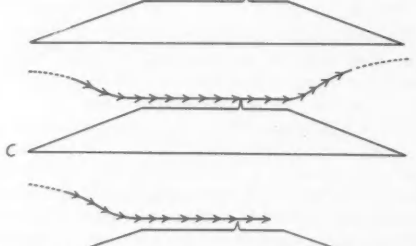

Fig. 2. Diagram to illustrate the union of α-amylase to its substrate.

But when a chain of less than 9 units has to be split it is forced to 'engage' only a part of the specific zone. Also the end of a long chain may fall by chance over the specific zone and unite as in the diagram at *D* without occupying all of the 9 available spaces. Union in such cases is assumed to be less easy, and fission to be much slower than in the previous case. With less glucosidic units engaged on either side of the point of fission there results a weakening of the hold and a lessening of the 'tension' which can be brought to bear.

We have assumed the point of fission to be three units from the one end of the specific zone of the enzyme, which in the case of our bacterial α-amylase would best explain the facts. In this case a fission of one glucosidic unit would be more difficult than that of two, and two more difficult than that of three units or more. A short chain of, for example, six units might (but would not necessarily) unite more readily as at *E* than as at *F* since in the position *E* a hold of two units on the one side of the fission point is secured even though this entails sacrifice of a one-unit hold on the other side. When a long chain is united in this way, but overlaps the specific zone at the other end, as at *D*, this sacrifice would not be incurred. In this case the chance of union and speed of fission as at *E* may exceed those of union *F* even more than they do with the short chain, since the long chain, while gaining an additional hold on one side, loses nothing on the other because all holds there are taken up in both cases. Thus the fission of one unit from the reducing end of a chain of 9 or more than 9 units length would be relatively rare.

On this hypothesis, fission of the links in long chains would be random, except for a limited number of links at each end. But fragments of 8 units and less tend to select their position on the enzyme and in consequence undergo fission in a more planned manner, depending on the source of the enzyme. For instance in the case of malt α-amylase, action as at *E* is the most common. However, fission at other links may take place excepting, of course, the link next to the non-reducing end, which is always sacrosanct to the amylases. Fission such

as *G* can never take place. The glucose group at the non-reducing end apparently cannot 'engage'. The exceedingly slow fission of maltotriose and non-fission of maltose follow from the above hypothesis. With salivary amylase the fission point would also be as at *E*, because there are holding places for two glucose units between the fission point and the end as against three such places with the bacterial amylase. However, with salivary amylase the number of units engaged on the other side of the fission point is only three (possibly four) as against six for bacterial and malt α-amylases.

It will be remembered that the shape of the chain is helical and that the six units to the left of the fission point (at *AB*) would be arranged in this way, occupying about one coil of the helix. The relative immunity from fission by the malt and bacterial amylases shown by the linkages in this coil is presumably associated with the need for the whole coil to unite with the receptive part of the enzyme to effect a firm union. Even in the case of salivary amylase five units (possibly six) in all are involved, amounting almost to one coil.

This hypothesis involves a random fission with exceptions in a number of linkages, especially at the non-reducing end, and consideration must now be given to the bearing of the experiments on this.

Action of the bacterial and malt α-amylases on amylose

To arrive at the achroic stage (at which no chains have greater length than 8 units) by the most economical fission of amylose, a planned attack by the enzyme on every eighth link would be needed, and the product would be achroic at R_m 25. By a purely random fission this would not be achieved at 3 times this value of R_m. In Tables 1 and 2 the achroic stage was reached in dilute solution at R_m 27–30, corresponding to mean chain length of about 7. Chromatograms (Table 4, Fig. 1) confirmed the relative abundance of dextrins of chain lengths 6, 7 and 8. However, this does not necessarily mean that the enzyme had primarily split the helical chains at regular intervals, say one turn or one coil apart. If the enzyme splits each coil at the corresponding link we should expect fission products equal to one coil each and all having the same chain length (either 6 or 7). The specific union of enzyme and substrate envisaged for this must essentially be associated with the helical structure of the substrate. But the same enzyme is also able to combine with a short saccharide chain such as hexasaccharide (also curved or part of a coil in shape) and to split from it glucose, maltose and maltotriose. For the latter purpose the enzyme needs to combine with the substrate molecule at all or most of the points along the chain. Such union and fission, if applied to the long helical chain, would be expected to result in the latter being split at various points, not necessarily one per coil, at uniform intervals and a more random type of fission would be expected. The variability in the chain length of the achroic dextrins (Table 4) also is more compatible with a random fission of the links in a long chain. There are, however, the important qualifications already discussed, namely, that in the case of the malt and bacterial amylases five links at one end and one or more at the other end of a chain are less readily split than the remainder. Assuming such qualification, it can be seen how it comes about that the fission products at the achroic stage are mostly of 6–8 units chain length. During the dextrinization phase with bacterial α-amylase, chains of 12 units or more can be split into parts of 6 or more. As the reaction progresses, chains of 6–11 units chain length increasingly appear. These are too short to give rise to two fission products of 6 units or more. Those of 9–11 units chain length are now split into, e.g. a hexasaccharide, and a short chain. Those of 6–8 units chain length are already achroic and are only split very slowly (secondary fission) as already discussed. In this example, from 6 chains, one each of 6–11 units chain length, respectively, are thus formed, six of chain length 6–8 and one each of chain length 2, 3 and perhaps 4 units each. Thus, at the end of the dextrinization phase, fission products of 6, 7 and 8 units chain length are the prevailing ones, and shorter chains of 2, 3 and perhaps 4 units chain length form a minority. The dextrin used in Fig. 1, itself a bacterial amylase fission product, yielded fragments of unit chain length 6, 7 and greater. The achroic fission products of amylose by the malt and bacterial enzymes were shown to contain both 6 and 7 units chain length by separation on a charcoal column followed by the action of β-amylase (Table 4).

The next point to be noted is that small but significant amounts of maltotriose, maltose and even glucose are formed during the dextrinization period even when secondary fission is minimized by the use of very dilute reaction mixtures (Tables 2–4). This indicates that the three linkages at one end of a long chain, if not both ends, are susceptible to fission even if this occurs less frequently than fission of the linkages further in the interior. As such linkages can be attacked when hexasaccharide is the substrate this is not surprising. Nevertheless, such linkages are split relatively slowly by the malt and bacterial amylases. From long chains the fission of glucose, for instance, is very much less than would be the yield by random fission. This is still true even if we take half the latter, because it is apparent that no splitting off of glucose takes place at the non-reducing ends. When bacterial α-amylase had split 0·041 % amylose to R_m 18 no evidence of glucose could be obtained by paper chromatography, indicating that less than

0·04 % had been formed (Table 3). Random fission would have yielded 0·8 % by R_m 18. Even in 0·84 % amylose, at R_m 30·6 (Table 3), glucose formed at most 0·5 % against 2·25 % theoretically producible by random fission. Malt α-amylase produced less than 0·2 % glucose in splitting 0·025 % amylose to R_m 22·8 (theoretical for random fission, 1·3 %). This was formed well before the achroic stage and could not have been split from short-chain fission products, but was derived from longer chains.

The fission of maltose and maltotriose from amylose cannot begin or is negligible until some reducing chain ends have been formed. There must be a lag, but as more primary fissions occur to render reducing ends available, the splitting off of maltotriose, maltose and, on a smaller scale, glucose begins. The rate of liberation of these sugars would start from zero and increase continuously. This increase would be roughly in arithmetic progression if the reaction as a whole (i.e. fission of all linkages attacked) were of zero order. Somogyi's (1940) results for starch as substrate and urinary amylase are in general agreement with this, but whereas Somogyi considered glucose to be split only from short-chain fission products, it is here contended that glucose can be split from the reducing ends of all fission products, whether short or long chains. Such fission, however, is slower than that of the linkages which are split at random, while fission of the end link at the non-reducing end never takes place with the α- and β-amylases. The very slow fission of maltotriose is illustrated by Tables 8 and 9. Inhibition of amylolysis by maltose was a contributory factor. Chromatograms have also shown that the stages of secondary fission by the malt and bacterial α-amylases at about R_m 90 include that of the tetraose.

The malt and bacterial amylases used here may therefore be taken only to attack with great difficulty the second to the fifth linkages from the non-reducing end, and the first or first two linkages as the case may be at the reducing end. Within these points other linkages are attacked at random.

Action of salivary amylase on amylose

Salivary amylase exhibits many features in marked contrast to malt and bacterial α-amylases. With it, fission of the first-formed achroic products proceeds relatively quickly and much maltose, maltotriose and tetraose are formed before the achroic stage is reached. On the other hand, less material of chain length 6–8 units is present at this stage than with malt and bacterial amylases (Table 4, Fig. 1). Whereas with bacterial amylase the minimum length of chain normally split from a non-reducing end is 6, with salivary amylase it is 3. The maximum lengths for resistance to fast fission are 8 and 4, respectively. Just as the prevalence of saccharides of 6–8 units chain length at the achroic stage is consistent with random fission by the bacterial amylase, so is the prevalence of 3–4 units chain length in the case of salivary amylase, in each case with the reservations postulated.

Roberts & Whelan (1951) and Whelan & Roberts (1952) have concluded that all the linkages in the amylose chain except one at each end are equally liable to fission by salivary amylase. Their final yields of maltose and maltotriose from amylose were in good agreement with the relative quantities (in moles, 2·35 : 1) calculated on this assumption. Our observations conflict with this conclusion so far as the second linkage from the non-reducing end is concerned. But if this is split very much more slowly than the others, while the others are split at random, the final yield of maltose would be less than that found by Roberts & Whelan. If, however, the third linkage, although split much faster than the second, is also split more slowly than the remaining susceptible linkages, the yields obtained could still be accounted for. This would mean that not only the end linkages but the second and third from the non-reducing end would be exceptions to the random attack.

General

Meyer & Gonon (1951) state that when a given fraction of linkages in amylose have been split, the proportions of the various fission products depend, not on the source of the α-amylase or of the amylose, but on the degree of fission. They cite results with the malt and pancreatic enzymes, the latter of which acts like salivary amylase (Bernfeld, Staub & Fischer, 1948). However, Table 4 and Fig. 1 clearly show that malt and salivary amylases differ in this respect near the achroic stage. The identity of action among α-amylases which has been claimed by these workers on the basis of identical ratios, saccharifying power/dextrinization power, may indeed be misleading. The early phases in which the necessary determinations were made are presumably sufficiently similar for the various α-amylases to give this effect because most of the fission is at random. But with increasing numbers of non-reducing ends, the number of linkages which are relatively immune to this or that enzyme becomes a controlling factor in determining where fission will take place.

SUMMARY

1. The α-amylases of malt and *Bacillus subtilis* hydrolyse amylose ultimately to maltose and glucose. The glucose is not derived wholly by fission of maltotriose.

2. At sufficient dilution the action of these amylases can be almost confined to dextrinization, with negligible fission of the achroic dextrins, which are of chain length 6–8 units.

3. Nevertheless, under these conditions, some maltotriose, maltose and glucose are formed by fission of longer chains. The yield of glucose is far below that expected from a random fission.

4. The action of these enzymes on dilute amylopectin yielded, in the early stages, negligible traces of fission products of shorter chain length than 6–8 units. Salivary and fungal (*Aspergillus oryzae*) amylases under the same conditions yielded products of chain length of 3 units and upwards.

5. Dilute amylose in corresponding reactions yielded appreciable quantities of fission products of chain length of 2 units and upwards (malt and salivary) or 3 units and upwards (bacterial and fungal).

6. Salivary amylase does not readily attack the first two, and the bacterial and malt α-amylases the first five linkages from the non-reducing end.

7. Salivary and malt α-amylases readily attack the second and bacterial amylase the third linkage from the reducing end, other linkages nearer this end with increasing difficulty. It is concluded that linkages other than those near the ends here specified are attacked at random.

8. The action of the α-amylases on shorter linear chains, such as maltohexaose and maltotriose, is restricted as regards linkages near the non-reducing end so that fission must often take place nearer to the reducing end than is normal, and more glucose may be formed.

9. A hypothesis is suggested to explain the actions of the α-amylases.

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The Purification of Aconitase

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The enzyme which catalyses the reaction citric acid ⇌ *cis*-aconitic acid ⇌ *iso*citric acid was discovered by Martius & Knoop (1936), and named aconitase by Breusch (1937). Although this enzyme has been known for some 16 years, it has not been isolated in the pure state. Ochoa (1948) was able to concentrate the enzyme by ammonium sulphate fractionation, but there was no increase in the specific acitivity and no further attempts were made to purify it. Buchanan & Anfinsen (1949) obtained a 23-fold purification of aconitase by low-temperature ethanol and ammonium sulphate fractionation. Electrophoretic analysis showed that the preparation consisted of 'three non-homogeneous components', and the purity of the enzyme was estimated to be 30%.

The purification of aconitase has been greatly hampered by its apparent instability. Krebs & Eggleston (1944) found that glycerol stabilized crude enzyme extracts, but Buchanan & Anfinsen (1949) reported that glycerol was without effect in stabilizing purified preparations. They also found

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that whilst cysteine stabilized the crude enzyme, it strongly inhibited the purified enzyme. Citrate or *cis*-aconitate were found to be the most effective stabilizers of aconitase.

An important finding for the further study of the purification of aconitase was made by Dickman & Cloutier (1950). They found that crude solutions of aconitase could be both stabilized and activated by the addition of Fe^{2+} and cysteine. Further work (Dickman & Cloutier, 1951) showed that these agents were also effective in stabilizing and activating more highly purified solutions of the enzyme. Fe^{2+} was the only cation which gave consistent activation of aconitase. Ascorbic acid was as effective as cysteine as a reducing agent, whilst glutathione was only one-half as effective.

As part of a project to extend the preliminary investigations of Peters & Wilson (1952) on the effect of fluorocitrate on aconitase, an attempt was made to purify the enzyme further in the light of the findings of Dickman & Cloutier (1950, 1951). For a preliminary communication see Morrison (1953).

EXPERIMENTAL

Reagents. *cis*-Aconitic anhydride (m.p. 77°) was prepared from *trans*-aconitic acid by the method of Malachowski & Maslowski (1928). Sodium *cis*-aconitate was formed by neutralizing the anhydride to pH 7·4 with N-NaOH. DL-*iso*Citric acid (m.p. 136°) was prepared by hydrolysis of the lactone. Trichloromethylparaconic acid was prepared by the method of Fittig & Miller (1889) and converted into *iso*-citric lactone by the method of Krebs & Eggleston (1944). Sodium *iso*citrate was formed by neutralizing the acid to pH 7·4 with N-NaOH. Both compounds were prepared by Mr R. W. Wakelin. The ethanol was taken to be 100%. L-Cysteine hydrochloride used in the later work was free from iron and obtained from Roche Products Ltd. The ammonium sulphate was British Drug Houses Ltd. Special Grade, and 2:2′-dipyridyl was a British Drug Houses Ltd. Spot Test reagent. All other reagents were A.R.

Estimation of citrate. The method of Pucher, Sherman & Vickery (1936), as modified by Buffa & Peters (1949), was used. Samples ranging from 1·0 to 3·0 ml. and containing up to 200 μg. of citric acid were used, as within this range the colour of the final solution was proportional to the amount of citric acid present.

Estimation of protein. The protein concentration of aconitase solutions was determined by measurement of the absorption in the Beckman spectrophotometer at a wavelength of 280 mμ. after the appropriate dilution with 0·1 N-NaOH. Under these conditions, a solution containing 1 mg. protein/ml. was found to have an absorption of 1·5.

Activation of aconitase. A suitable sample of the enzyme solution was measured into a test tube and placed in an ice bath. Non-neutralized ferrous ammonium sulphate and neutralized cysteine were added, so that on dilution of the solution with cold water to 10 ml., the final concentration of Fe^{2+} was 5×10^{-4} M and cysteine was 0·01 M. The mixture was then neutralized with N-NaOH to pH 7·4, using a glass electrode, and incubated in the ice bath for 1 hr. before the aconitase activity was determined. Except where otherwise stated, the various fractions were activated before an activity determination was carried out.

Determination of aconitase activity. To a series of Pyrex test tubes, 4·4 ml. or 4·3 ml. of water were added; 0·5 ml. of a solution containing 20 μmoles of *cis*-aconitate at pH 7·4 was then added and the test tubes were placed in a water bath at 30°. After an equilibration period of 5 min., the reaction was started by the addition of 0·1 ml. or 0·2 ml. of enzyme solution. The reaction was stopped after 15 min. by the addition of 0·5 ml. of 50% (w/v) trichloroacetic acid. If a visible precipitate formed, it was filtered off through a no. 30 Whatman filter paper, otherwise the citrate estimation was carried out on the acidified solution without further treatment. It was found that small amounts of protein did not interfere with the estimation. Under these conditions, the amount of citric acid formed from *cis*-aconitic acid was proportional to the amount of enzyme added. The activity determinations were carried out at two enzyme levels to ensure that the substrate concentration was not limiting.

Definition of a unit of aconitase activity. One unit of aconitase activity was taken to be the amount of enzyme which formed one μmole of citric acid from *cis*-aconitic acid in 15 min. at pH 7·4 and 30° in the absence of buffer.

Specific activity. This was taken to be the units of aconitase activity/mg. of protein.

Other enzyme activity determinations. *iso*Citric dehydrogenase activity was determined according to the method of Ochoa (1951). One unit was defined as a change of log I_0/I of 0·01 per min. Fumarase activity was determined by the method of Racker (1950). One unit was defined as a change of log I_0/I of 0·001 per min.

RESULTS

Activation of aconitase

As the activation of aconitase by Fe^{2+} and cysteine was of importance, the work of Dickman & Cloutier (1951) was repeated using their purified preparation of aconitase. The results were in good agreement with those obtained by these authors. However, the activating effect of cysteine alone is open to criticism. It was found that the preparation of cysteine which gave similar results to those of Dickman & Cloutier (1951) contained appreciable amounts of iron. Nevertheless, the importance of Fe^{2+} and cysteine as activators of aconitase was confirmed. The cysteine used in the later experiments described here was completely free from iron.

Inhibition of aconitase

Having confirmed that Fe^{2+} was an integral component of the system, it seemed likely that the presence of ions, such as phosphate, which are capable of forming insoluble complexes with iron, would lead to inactivation of the system as a whole. In order to show this experimentally, the same enzyme preparation was activated and pre-incubated at 30° with 0·05 M phosphate buffer (pH 7·4) for various periods of time. Substrate was then added and the reaction allowed to proceed for

15 min. In the control system, water replaced the buffer. Fig. 1 shows that phosphate caused a rapid inactivation of the activated enzyme when substrate was absent. When phosphate and substrate were added simultaneously, the enzyme activity was greater than that obtained in the absence of phosphate. This increased enzyme activity was shown to be due to the influence of the ions present in solution on the pH optimum of the enzyme. The enzyme activity was not increased by the simultaneous addition of excess of Fe^{2+} (5×10^{-3} M) and substrate, following pre-incubation for various periods in the presence of phosphate. Thus, phosphate is capable of inactivating the activated enzyme. This finding is in contrast to the results of Dickman & Cloutier (1951), for they found that phosphate did not decrease the activity of an activated preparation. As similar results were obtained with veronal—acetate, borate and glycerophosphate buffers at pH 7·4, enzyme solutions were not diluted with buffers and activity determinations were carried out in the absence of buffer. A check of the pH of the system before and after incubation showed that although there was a small alteration of pH in the absence of buffer, the results were perfectly reproducible.

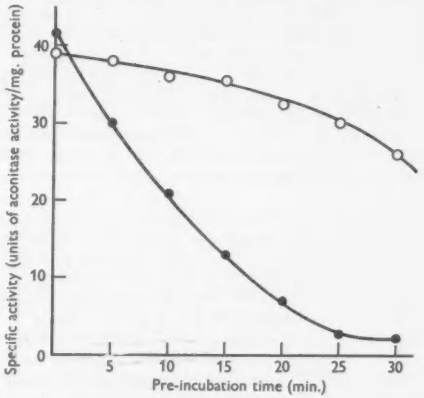


Fig. 1. Effect of incubating aconitase in the absence of substrate. The enzyme was activated with Fe^{2+} and cysteine and incubated at 30° for various periods of time. *cis*-Aconitate was added and the reaction was allowed to proceed for 15 min. ○—○, no buffer present, reagents adjusted to pH 7·4; ●—●, 0·05 M phosphate buffer (pH 7·4).

The inactivating effect of phosphate in the absence of substrate can at least partially explain the instability of the enzyme found by Krebs & Eggleston (1944) and by Peters & Wilson (1952). These workers used phosphate buffer in the test systems. It also renders the assay figures of Johnson (1939) of the aconitase activity of various animal organs open to criticism, as the organs were extracted with phosphate buffer. Phosphate was therefore not used during the course of the purification of the enzyme.

Preparation of aconitase by the method of Buchanan & Anfinsen (1949)

As Fe^{2+} and cysteine had not been used by Buchanan & Anfinsen (1949) in their test system for aconitase activity, it was probable that their final enzyme preparation was, in fact, of a higher specific activity than reported. In order to determine whether or not this was the case, aconitase was prepared according to the method of these workers. A study was then made of the activity of various fractions in the presence and absence of Fe^{2+} and cysteine. Although Buchanan & Anfinsen (1949) carried out their tests at 38°, in the present work the tests were made at 30° and the activities were multiplied by 1·5, the temperature coefficient found for 38°/30°.

The results of Table 1 clearly show that in the absence of Fe^{2+} and cysteine, the specific activities of the intermediate fractions are much lower than those claimed by Buchanan & Anfinsen (1949). It was only in the presence of these two agents that

Table 1. *Comparison of aconitase activities in the presence and absence of* Fe^{2+} *and cysteine*

(Fractions were prepared according to the method of Buchanan & Anfinsen (1949). Activation by Fe^{2+} and cysteine was carried out as described in the text. Temp. 30°; pH 7·4. The results obtained were multiplied by 1·5, the temperature coefficient for 38°/30°, so that they were equivalent to activities at 38°. The specific activity is the units of aconitase activity/mg. protein.)

	Results of Buchanan & Anfinsen (1949)		Present results				
	No additions		No additions		Fe^{2+} + cysteine added		
Fraction	Total units	Specific activity	Total units	Specific activity	Total units	Specific activity	Ratio of activities of activated enzyme / non-activated enzyme
Extract	310 000	10·0	261 000	10·5	278 000	11·3	1·07
First ethanol ppt.	133 000	18·9	144 000	26·9	220 500	41·1	1·53
Second ethanol ppt.	102 000	68	100 500	29·3	175 000	51·5	1·74
Third ethanol ppt.	63 000	132	45 000	45	105 000	107	2·35

similar activities could be obtained. A comparison of the activities of the activated and non-activated fractions shows that the degree of activation by Fe^{2+} and cysteine increases as purification proceeds, indicating that the prosthetic group of the enzyme is removed to some extent by the fractionation procedure. It will also be noted that the relative purification of the enzyme in each step differs markedly from that obtained by Buchanan & Anfinsen (1949). Results for the ammonium sulphate step are not given, as it was found that the enzyme was completely precipitated when the solution was brought to 66% saturation. There was no increase in the specific activity.

Method of isolation

The following fractionation procedure was adopted after repeated trials. The superscripts refer to the notes given later.

Extraction of tissue. Immediately after the death of the pig, the heart was removed, cut into strips of about 0·25 in. thickness, freed from fat and connective tissue and placed in ice. At the laboratory, the hearts were minced through a coarse mincer and stored at −15°. Under these conditions, pig heart could be stored for some months without loss of aconitase activity. A 1 kg. sample of frozen heart was allowed to thaw at room temperature and then comminuted in 200 g. lots for 2 min. in a pre-cooled Waring Blendor with 600 ml. of 0·004 M citrate buffer (pH 4·7) and 130 ml. of $CHCl_3$.[1] The product was centrifuged at −1° for 15 min. at 1000 *g*. The clear red-amber supernatant (pH 5·7)[2] was poured off and filtered through a fluted filter paper (Whatman no. 531).

First ethanol fractionation. For convenience, the supernatant was divided into two equal amounts and fractionated simultaneously. From this stage onwards, all operations were carried out in the coldroom. The supernatant was brought to 0° in a dry-ice : ethanol bath and 90% (v/v) ethanol[3] was added with mechanical stirring at the rate of 250–300 ml./hr., to a concentration of 15% (v/v). During the addition of ethanol, the temperature was lowered to −5°.[4] Hyflo Supercel was added to the solution (5 g./l.) and filtration was carried out on a Büchner funnel under slight pressure (Whatman no. 30 filter paper). To the clear red-amber filtrate, saturated NaCl (1·25 ml./100 ml. of filtrate) was added. The ethanol concentration of the solution was brought to 45%, the temperature being gradually lowered to −10°. The mixture was allowed to stand overnight at −10°.[5]

Second ethanol fractionation. The precipitate was centrifuged off at −10° and stored at this temperature, whilst another 1 kg. of pig heart was treated in the same fashion. The two precipitates were combined and dissolved in 800 ml. of ice-cold water. A small amount of insoluble matter was centrifuged off to yield a clear deep red-amber solution. The solution was brought to 0° and ethanol added to a concentration of 10% (v/v),[6] the temperature being lowered to −4°. A small amount of a white precipitate was centrifuged off at −4°. The pH of the supernatant, which was about 5·6, was adjusted to pH 6·8 with 5% (w/v) sodium carbonate solution. Ethanol was added to a concentration of 23% (v/v), whilst the temperature was lowered to −10°. The precipitate was centrifuged off at −10° and dissolved in 100 ml. of ice-cold water.

Ammonium sulphate fractionation. The aqueous solution was diluted 2·5 times with 0·125 M citrate buffer (pH 6·8). $(NH_4)_2SO_4$ (36·7 g./100 ml. of solution) was added slowly, the temperature being lowered to −6°. The yellow precipitate was centrifuged off at −6° for 10 min. at 5000 *g*, or alternatively filtered on a Büchner funnel with the aid of Hyflo Supercel. To the supernatant, $(NH_4)_2SO_4$ (9·2 g./100 ml. of supernatant) was added slowly and the temperature was lowered to −10°. The precipitate was centrifuged off at −10° for 15 min. at 5000 *g* and dissolved in 90 ml. of 0·004 M citrate buffer (pH 5·7) to give a deep-amber solution. This solution was dialysed overnight with stirring against the same buffer.[7]

Heat fractionation. The dialysed solution in 20–25 ml. lots was heated in a water bath for 15 min. at 50° and cooled rapidly in an ice bath. The white flocculent precipitate was centrifuged off at 0° and discarded.

Ethanol fractionation at pH 8·0. The supernatant was cooled to 0° and ethanol was added to a concentration of 20% (v/v), the temperature being lowered to −5°. A small precipitate was centrifuged off at −5° and discarded. The supernatant was adjusted to pH 8·0 with a saturated solution of $NaHCO_3$ and ethanol was added to a concentration of 50% (v/v).[8] During the addition, the temperature was lowered to −12°. The reddish precipitate was centrifuged off from a cloudy supernatant (due to the presence of $NaHCO_3$) at −12° and dissolved in 20 ml. of 0·004 M citrate buffer (pH 5·7).

Ammonium sulphate fractionation at pH 8·5. After adjustment of the solution to pH 8·5 with ammonia, a saturated solution of $(NH_4)_2SO_4$ (pH 8·5)[9] was added dropwise until the saturation was 0·65. The temperature was gradually lowered to −10°. The precipitate was centrifuged off at −10°, dissolved in 10 ml. of 0·004 M citrate buffer (pH 5·7) and dialysed against the same buffer overnight at 0°. The final solution was amber in colour.

Notes. (1) $CHCl_3$ reduced the haemoglobin content of the extract. There was no denaturation of the enzyme and the specific activity of the extract was increased.

(2) Marked loss of enzyme activity occurred when the pH of the extract was adjusted below pH 5·0.

(3) 90% (v/v) ethanol was used throughout the fractionation.

(4) It was important that the solution should not freeze at this stage, as freezing leads to loss of enzyme activity.

(5) Contact of the aconitase with ethanol for this period had no harmful effect, for the specific activity was the same whether the solution was centrifuged immediately, or allowed to stand overnight.

(6) The ethanol already present was neglected.

(7) The enzyme could be dialysed against citrate buffer without loss of activity, whereas large losses of activity occurred when the dialysis was carried out against water and salt solutions.

(8) During the fractionation at alkaline pH values, care was taken to prevent CO_2 from distilling over from the dry-ice : ethanol bath into the protein solution.

(9) A saturated solution of $(NH_4)_2SO_4$ at room temperature was neutralized with strong ammonia, so that on dilution of 1 in 5, the pH was 8·5.

Attempts to purify the enzyme further were unsuccessful. Large amounts of calcium phosphate

gel were required to adsorb the enzyme from solution between pH 5·7 and 6·8 and no differential adsorption or elution could be obtained. Although the enzyme could be stirred with butanol for 30 min. at room temperature, this treatment did not alter the precipitation pattern after further ethanol fractionation. No protein was precipitated by the nucleic acid precipitants manganese sulphate and protamine sulphate. Fractionation with acetone precipitated the enzyme between 30 and 50% (v/v) acetone concentration, but marked loss of activity occurred even though the fractionation was carried out between $-5°$ and $-10°$. This finding is in contrast to that of Buchanan & Anfinsen (1949), but is consistent with the fact that acetone powders of pig heart are devoid of aconitase activity. The enzyme was not precipitated by sodium chloride or ammonium acetate. Ethanol fractionation in the presence of zinc was ineffective, presumably because the zinc reacted with the citrate present.

Although the enzyme constituted the major portion of the final solution, it could not be induced to crystallize. The slow addition of ethanol always gave rise to an amorphous product. Attempts were also made to crystallize the enzyme from ammonium sulphate solutions by the addition of an amount just insufficient to cause precipitation, followed by adjustment of the pH. Within the pH range 5·7–8·5, both in the presence and the absence of Fe^{2+} and cysteine, the enzyme precipitated only in small amounts and in an amorphous form.

In Table 2 is given a summary of the yields and degrees of purification of aconitase during the fractionation procedure. This procedure brings about a 24-fold increase in purity of aconitase, with an overall recovery of about 12%. The final product has a specific activity of about 265 when the test is carried out in the absence of buffer. If the test is carried out in 0·05 M phosphate buffer (pH 7·4), the specific activity is increased to 336. This increase is due to the fact that the pH optimum of aconitase is dependent upon the ions present in solution. The variation of the pH optimum in different buffers will be reported later. Thus, at 38° the specific activity is about 500, which corresponds to a value of 235 as claimed by Buchanan & Anfinsen (1949) for their final preparation.

General properties of aconitase

The enzyme in dilute citrate buffer at pH 5·7 retained its activity for many weeks when stored in the frozen state and was unaffected by repeated freezing and thawing. It was not found necessary to add Fe^{2+} and cysteine as stabilizing agents, although in the absence of both or either of these agents the enzyme activity was markedly reduced. (Details of the activation of aconitase will be reported later.) The final enzyme preparation could also be freeze-dried without loss of activity, but it was found more convenient to keep it in the frozen state. Fig. 2 shows that aconitase is stable to heat until a temperature of 51° is attained, after which there is a sharp loss of activity. A characteristic feature of the enzyme was its wide range of precipitation. Aconitase activity was found in every fraction obtained with all solvents and salts used over the pH range from 5·7 to 8·5.

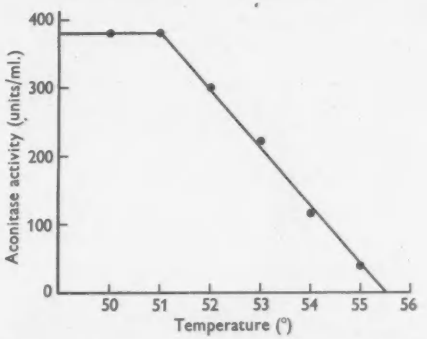


Fig. 2. The effect of temperature on aconitase activity. The protein concentration was 9 mg./ml. Heating was carried out in a water bath for 15 min., using 2·0 ml. samples of a solution of the dialysed $(NH_4)_2SO_4$ precipitate. Aconitase activity was determined after activation with Fe^{2+} and cysteine as described in the text. Temp. 30°, pH 7·4.

Table 2. *Summary of yields and specific activities of fractions obtained during the purification of aconitase from* 2 kg. *pig heart*

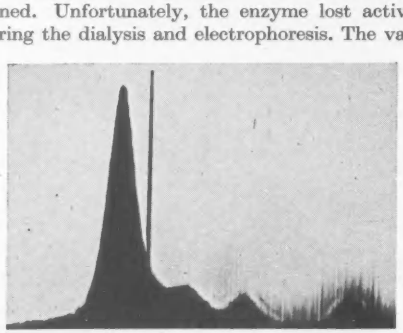
(The specific activity is the units of aconitase activity/mg. protein. For other details see text.)

Fraction	Volume (ml.)	Total protein (g.)	Total units	Specific activity	Yield (%)
Extract	6240	39·3	437 000	11·1	100
First ethanol ppt.	832	5·3	260 000	49	60
Second ethanol ppt.	112	2·2	212 000	96	49
Dialysed ammonium sulphate ppt.	116	0·98	157 000	168	36
Supernatant after heating at 50°	102	0·72	135 000	188	31
pH 8·0 ethanol ppt.	25	0·41	96 000	232	22
pH 8·5 ammonium sulphate ppt.	15	0·19	50 400	265	12

Electrophoretic analysis

The electrophoretic pattern of the final preparation of aconitase is illustrated in Fig. 3. All the components migrated to the anode when electrophoresis was carried out at pH 8·6, but at pH 5·7 at least two of the minor components still migrated to the anode whilst the main component migrated to the cathode. pH values below 5·0 could not be used owing to denaturation of the enzyme.

In order to determine whether or not the main component was responsible for the aconitase activity, a second electrophoretic run was carried out after dialysis of a 1% solution of the protein against veronal buffer at pH 8·6 (ionic strength 0·075) which contained sodium citrate (ionic strength 0·025). A similar electrophoretic pattern was obtained. Enzymic analysis of the main component after separation showed that this component was responsible for the aconitase activity, whilst the other components were devoid of activity. Both fractions were dialysed overnight against 0·004 M citrate buffer (pH 5·7) and activated with Fe^{2+} and cysteine before the aconitase activity was determined. Unfortunately, the enzyme lost activity during the dialysis and electrophoresis. The value obtained for the specific activity of the electrophoretically homogeneous protein was less than that of the original solution. The main component was completely free from the amber pigment which was associated with the enzyme during the stages of purification. The mobility of the amber protein was somewhat similar to that of aconitase, so that electrophoresis had to be continued for a long period to effect separation. It may be calculated that the purity of the electrophoretically homogeneous protein which possesses aconitase activity in this preparation is 75–80%. If this component consists entirely of aconitase, it would appear that aconitase represents a large proportion of the total protein of heart muscle.



(*a*)

(*b*)

Fig. 3. Electrophoretic patterns of the aconitase preparation obtained after fractionation as described in the text. Veronal buffer: pH 8·6; ionic strength, 0·1; temperature, 1°; current, 10 mA; protein concentration, 1%; time, 168 min. (*a*) Descending boundary; (*b*) ascending boundary.

Conversion of cis-*aconitate and* iso*citrate to citrate*

Jacobsohn, Soares & Tapadinhas (1940), on the basis of kinetic measurements, have postulated that aconitase might consist of two distinct enzymes, α-aconitase catalysing the reaction *cis*-aconitate ⇌ *iso*citrate, and β-aconitase catalysing the reaction *cis*-aconitate ⇌ citrate. Martius & Leonhardt (1943) questioned this finding, and up to the present time there has been no definite evidence for the existence of two aconitases. Buchanan & Anfinsen (1949) found that the ratio of the activity of the enzyme in forming citrate and *iso*citrate from *cis*-aconitate remained practically constant during the fractionation procedure. They also found that the two activities were not separated by electrophoresis. However, the active fraction was contaminated by a coloured protein which was considered to be a haemoglobin or myohaemoglobin or both.

In this work it has been shown that the coloured component does not possess aconitase activity. The electrophoretically homogeneous protein which showed aconitase activity was devoid of colour and was capable of converting both *iso*citrate and *cis*-aconitate into citrate. Thus it is established that a single protein which is homogeneous in the electrophoresis apparatus can bring about those reactions associated with aconitase activity. This finding is consistent with the hypothesis advanced by Ogston (1951) as a result of theoretical considerations. It is, of course, possible that the protein is not homogeneous in the ultracentrifuge and this point will be investigated later.

Activation of aconitase fractions by Fe^{2+} *and cysteine*

During the purification, various fractions were tested for the ability to convert both *iso*citrate and *cis*-aconitate into citrate. Table 3 shows that the ratio of the two activities remains constant within the limits of experimental error. The addition of Fe^{2+} and cysteine increased the two activities equally, so that there was no alteration in the ratio. The activating effect of Fe^{2+} and cysteine during the

Table 3. *Comparison of the rates of citrate formation from* isocitrate *and* cis-*aconitate by activated and non-activated fractions of aconitase*

(Fractions were obtained and activated as described in the text.)

	Specific activities (units of aconitase activity/mg. protein)						Ratio of activities of activated enzyme / non-activated enzyme	
	Non-activated			Activated				
Fraction	(*a*) *cis*-Aconitate	(*b*) *iso*-Citrate	Ratio *a* : *b*	(*c*) *cis*-Aconitate	(*d*) *iso*-Citrate	Ratio *c* : *d*	*cis*-Aconitate	*iso*-Citrate
Extract	7·0	4·35	1·61	11·4	7·2	1·59	1·63	1·66
First ethanol ppt.	31	17·5	1·78	49	31	1·58	1·6	1·8
Second ethanol ppt.	50	32·5	1·54	96	56	1·71	1·9	1·7
Ammonium sulphate ppt.	72·5	45·5	1·59	151	97	1·56	2·1	2·1
Dialysed ammonium sulphate ppt.	7·0	4·7	1·49	142	85	1·67	20	18
Supernatant after heating at 50°	6·7	4·2	1·60	174	113	1·54	26	27

early stages of purification was small and remained reasonably constant. After dialysis the activation was greatly enhanced. Fe^{2+} and cysteine can, therefore, completely replace the dialysable prosthetic group. The addition of 10^{-4} M 2:2′-dipyridyl to the dialysis medium caused the formation of ferrous tridipyridyl inside the dialysis sac, and this complex slowly diffused out into the medium. No tests have, as yet, been carried out for the identification of a reducing substance in the dialysis medium. It would appear that not only is Fe^{2+} capable of activating aconitase, but it is also the metal with which the enzyme is associated *in vivo*. The results also suggest that the Fe^{2+} is bound to the enzyme sufficiently strongly to resist splitting as a result of ethanol and ammonium sulphate fractionation, but the linkage is readily broken by dialysis.

Tests for the presence of other enzymes in the final preparation

The final preparation contained 196 units of *iso*citric dehydrogenase activity/mg. of protein, and 1100 units of fumarase activity/mg. of protein. No evidence was found for the presence of lactic dehydrogenase. Thus it is possible that two of the three minor components of the electrophoretic pattern are due to the presence of *iso*citric dehydrogenase and fumarase.

SUMMARY

1. The activation of aconitase by Fe^{2+} and cysteine has been confirmed. Phosphate was shown to inactivate rapidly the activated enzyme in the absence of substrate.

2. Aconitase has been purified 24-fold by low-temperature ethanol and ammonium sulphate fractionation combined with heat fractionation. The purity of the final preparation was estimated to be 75–80%.

3. The final aconitase preparation was contaminated to a small extent by *iso*citric dehydrogenase and fumarase.

4. Aconitase has been isolated from the final preparation as an electrophoretically homogeneous protein. This protein was free from pigment and was capable of converting both *iso*citrate and *cis*-aconitate into citrate.

5. During the purification, the degree of activation of aconitase by Fe^{2+} and cysteine increased, especially after dialysis.

It is a pleasure to express my thanks to Sir Rudolph Peters, F.R.S., for his advice and interest in the work. Thanks are also due to Dr E. O. Field for carrying out the electrophoretic analyses of the enzyme preparations and to Miss S. Read for skilled technical assistance. This work was carried out during the tenure of an Australian National University scholarship.

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Studies on the Determination of the Sequence of Amino Acids in Peptides and Proteins

2. THE SEPARATION OF THE METHYL ESTERS OF *N*-2:4-DINITROPHENYL DERIVATIVES OF AMINO ACIDS BY ADSORPTION CHROMATOGRAPHY AND THE FREE AMINO GROUPS OF INSULIN

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N-2:4-Dinitrophenylamino acids were first prepared by Abderhalden & Blumberg (1910). Sanger (1945) showed that mixtures of these compounds could be conveniently separated by partition chromatography on silica columns. He then applied this method to determine the free amino groups of insulin.

Although the use of methods requiring extremely small amounts of material is advantageous for the study of many biochemical problems, it is necessary to use somewhat larger quantities in order to ascertain by physico-chemical means the identity and the homogeneity of chromatographically separated fractions. Our results on the chromatographic analysis of *N*-sulphazoyl derivatives of amino acids (Flowers & Reith, 1953) clearly demonstrated the necessity of such an examination.

As the columns used in partition chromatography are easily overloaded, we investigated adsorption chromatography on columns of activated alumina for the separation of *N*-2:4-dinitrophenyl (DNP) derivatives of amino acids. For this purpose we prepared the methyl esters of these derivatives, and examined their properties. Part of this work was communicated to the Biochemical Society (Lowther & Reith (Reich), 1949).

While this work was in progress, Mills (1952) and Green & Kay (1952) developed methods for adsorption chromatography of DNP-amino acids on kieselguhr and silica.

EXPERIMENTAL AND RESULTS

The preparation of methyl esters of N-2:4-dinitrophenyl derivatives of amino acids

N-2:4-Dinitrophenyl derivatives (DNP derivatives) were prepared by reaction of amino acids with 1-fluoro-2:4-dinitrobenzene (FDNB) by the method of Sanger (1945). They were then esterified either with methanol-HCl or with diazomethane in dioxan solution. Typical esterifications proceeded as follows. DNP-glycine (2·2 g.) was suspended in dry methanol (30 ml.), the suspension was saturated with dry HCl and refluxed for 10 min. After evaporation *in vacuo* the residue was dissolved in methanol and the solution repeatedly evaporated to dryness *in vacuo*. The dry residue was twice recrystallized from hot ethanol:light petroleum, b.p. 60–80° (1:1, v/v). Yield 1·5 g., m.p. 124–125°.

DNP-glycine (2 g.) was dissolved in dioxan (100 ml.). A freshly prepared solution of diazomethane (from 12 g. of *N*-nitrosomethyl urea) in ether (175 ml.) was added in five portions with shaking at each addition. The red solution was allowed to stand for 1 hr. at room temperature, and was then evaporated *in vacuo*. Yield, 2·1 g. of red crystals (100%). 0·218 g. was dissolved in benzene and purified on an alumina:benzene column. A thin, bright-red zone was retained at the top, and the broad, yellow band which moved down the column was collected, evaporated *in vacuo*, dissolved in dry benzene (2 ml.) and filtered to remove alumina. Evaporation *in vacuo* yielded 0·213 g. of yellow crystals, m.p. 124–125°.

The data on the DNP-amino acid methyl esters prepared in this way are given in Table 1.

αε-Di-DNP-L-lysine methyl ester. αε-Di-DNP-L-lysine (0·501 g.) was esterified by the methanol-HCl method. The preparation yielded a semi-solid material (0·498 g.) which would not crystallize. After chromatographic purification on an alumina:benzene column and elution with acetone, the *methyl ester* was obtained as a solidified oil melting between 80° and 100°. The substance had a very pronounced tendency to occlude solvent, which was difficult to eliminate. (Found: C, 48·0; H, 4·4; N, 15·5. $C_{19}H_{20}O_{10}N_6$, CH_3. CO. CH_3 requires C, 48·0; H, 4·7; N, 15·3%.)

ε-DNP-L-lysine methyl ester hydrochloride. ε-DNP-L-lysine HCl, H_2O (76 mg.) was suspended in methanol (5 ml.) previously saturated with dry HCl and cooled to room temperature. Dry HCl was then bubbled through the suspension, care being taken that the temperature did not rise noticeably above room temperature. After about 30 min., a fine, yellow precipitate appeared; HCl was allowed to pass for a further 20 min., and after 2 hr. at 0°, the yellow crystals were filtered off and dried *in vacuo* over conc. H_2SO_4 in the presence of solid KOH. The yield of *ε-DNP-L-lysine methyl ester hydrochloride* was 68 mg.; m.p. 144–147°. (Found: C, 42·6; H, 5·4; N, 15·5; Cl, 9·7. $C_{13}H_{19}N_4O_6Cl$ requires C, 43·0; H, 5·3; N, 15·4; Cl, 9·8%.) Solutions of this substance were heat-labile, and precautions taken in the estimation of the yield will be described under Chromatographic Separations.

Table 1. *DNP-Amino acid methyl esters prepared by esterification of DNP-amino acids with methanol-HCl*

N-2:4-Dinitrophenylamino acid				*N*-2:4-Dinitrophenylamino acid methyl ester			
						Analysis	
	M.p. (corr.) (°)	Yield (%)	M.p. (corr.) (°)	$[\alpha]_D^{18}$	Found (%)	Required (%)
Glycine	205	78	124 (ethanol)	—	C, 42·1; H, 3·6	$C_9H_9O_6N_3$ C, 42·3; H, 3·5
DL-Alanine	176	82	121 (ethanol)	—	C, 44·9; H, 4·3	$C_{10}H_{11}O_6N_3$ C, 44·5; H, 4·1
L-Alanine	177–8	65	80–81 (ethanol-light petroleum)	−26° (*c*, 1; 1 = 1 dm; toluene)	C, 44·6; H, 4·1; N, 15·4	$C_{10}H_{11}O_6N_3$ C, 44·5; H, 4·1; N, 15·6
DL-Aspartic acid	197–8 (decomp.)	77	110–111 (ethanol-light petroleum)	—	C, 43·7; H, 3·9	$C_{12}H_{13}O_8N_3$ C, 44·1; H, 4·0
DL-Methionine	118–20	40	81–2 (ethanol-light petroleum)	—	C, 44·2; H, 4·5; N, 12·4	$C_{12}H_{13}O_6N_3S$ C, 43·6; H, 4·6; N, 12·8
S-Methyl-L-cysteine	Oil	80	57–8 (ethanol)	−99° (*c*, 1; 1 = 1 dm; toluene)	C, 42·1; H, 4·4	$C_{11}H_{13}O_6N_3S$ C, 41·9; H, 4·2
L-Phenylalanine	185–7	56	109–111 (ethanol)	—	C, 55·6; H, 4·1; N, 12·0	$C_{16}H_{15}O_6N_3$ C, 55·6; H, 4·3; N, 12·2

Spectrophotometric estimations

A Unicam S.P. 500 Spectrophotometer was used. The DNP-amino acid methyl esters were estimated in ethanol solution (1 cm. cuvettes). The ethanol was always freshly purified by fractional distillation. For concentrations between 1·5 and 15 mg. of methyl ester/l. Beer's Law was found to be obeyed. Absorption spectra were taken between 200 and 420 mμ., and the molecular extinction coefficients (ϵ) were calculated at the main absorption maxima. The following values were obtained. DNP-glycine methyl ester: $\epsilon = 10300$ at 260 mμ.; 17200 at 342 mμ. DNP-L-phenylalanine methyl ester: $\epsilon = 9700$ at 260 mμ.; 17200 at 342 mμ. ϵ-DNP-L-lysine methyl ester HCl: $\epsilon = 8300$ at 260 mμ.; 16650 at 348 mμ.

The DNP-amino acids were estimated in $NaHCO_3$ solution (1%, w/v). The estimations were always performed immediately after the preparation of the solution. All the DNP-amino acids examined were perfectly stable in the $NaHCO_3$ solution for at least 3 hr. at room temperature. ϵ was calculated at the main absorption maxima (1 cm. cuvettes):

	265 mμ.	361 mμ.
DNP-glycine	8 800	17 100
DNP-alanine	8 800	17 100
DNP-L-phenylalanine	8 600	17 200
ϵ-DNP-L-lysine HCl, H_2O	8 500	17 700
	263 mμ.	355 mμ.
Di-DNP-L-lysine	15 850	30 850
	265 mμ.	353 mμ.
DNP-glycylglycine	9 020	16 000

For concentrations between 1 and 15 mg./l. Beer's Law was obeyed at 353, 355 or 361 mμ. At 263 or 265 mμ. slight discrepancies occurred.

Chromatographic separation of mixtures of DNP-amino acid methyl esters

Alumina was prepared by the method already described (Flowers & Reith, 1953). With this alumina it was possible to separate mixtures of methyl esters of DNP-amino acids into their individual components. For instance the chromatographic resolution of a mixture of the methyl esters of DL-alanine and *S*-methyl-L-cysteine was carried out in the following manner.

An alumina column (22 × 320 mm.) was prepared with a slurry of alumina in benzene:light petroleum, b.p. 60–80° (1:3, v/v). To a benzene solution (6 ml.) of the *S*-methyl-L-cysteine derivative (30·1 mg.) and the DL-alanine derivative (30·9 mg.), light petroleum (12 ml.) was added, and the solution was slowly passed through the column. The mixture of the coloured derivatives adsorbed on the top of the column was developed with benzene:light petroleum, b.p. 60–80° (1:3, v/v, 850 ml.), when two yellow zones formed which were clearly separated by a colourless zone (25 mm.). The yellow zones were separately eluted with a mixture of acetone and methanol (1:1, v/v), the two resulting solutions were filtered and concentrated *in vacuo*. The solution from the top zone gave 31·2 mg. of crystalline material, m.p. 55–6° (m.p. of the *S*-methyl-L-cysteine derivative 57°), while the bottom zone gave 31·1 mg. of crystalline material, m.p. 122° (m.p. of the DL-alanine derivative 121°).

The high yield, 104 and 103%, was found to be due to small amounts of alumina which in a finely dispersed form passed with the solution through the filter. It was found that this could be eliminated by dissolving the dry residue in a small amount of dry benzene, and filtering the benzene solution through a no. 4 sintered-glass filter. An experiment carried out in this way yielded a 100% recovery of the *S*-methyl-L-cysteine derivative ($[\alpha]_D^{18} = -100° \pm 2$; toluene) and a 98% recovery of the DL-alanine derivative (m.p. 121–122°).

Yields in the esterification of ε-DNP-L-lysine. The solution of the methyl ester of ε-DNP-L-lysine was heat-labile, and even evaporations *in vacuo* at room temperature decomposed a small amount of the compound. If the evaporations were performed at low temperature under standard conditions, the amount of the decomposition product was constant and very small. Its nature has not yet been investigated.

ε-DNP-L-lysine HCl, H_2O (76 mg.), esterified as described above, gave 68·0 mg. of ε-DNP-L-lysine methyl ester hydrochloride as yellow crystals and a yellow filtrate. The filtrate was evaporated *in vacuo* in the dark at room temperature, the residue dissolved in a small amount of methanol:$CHCl_3$ (5:95, v/v) and the solution purified chromatographically on an alumina column (22 × 320 mm.) prepared with benzene. The chromatogram was developed with methanol:benzene (2:98, v/v). An extremely narrow yellow zone remained strongly held at the top of the column (unreacted ε-DNP-L-lysine.HCl), a faint narrow zone was washed through the column, while the main broad, bright-yellow zone was eluted with methanol. The methanol solution was evaporated *in vacuo*, the residue dissolved in ethanol, and the amount of substance estimated spectrophotometrically. The amount found was 7·83 mg., thus the total yield from the esterification was 75·9 mg.

This seems to indicate a quantitative yield by the esterification procedure. This cannot be quite true since during chromatographic purifications of crude esterification products there was always a small amount of unreacted ε-DNP-L-lysine tightly held at the top of the column. The yield, however, is probably very nearly quantitative, the value of 101% being obtained because the crystalline product used as standard for spectrophotometry was not quite pure. The elementary analysis also points to that.

Yields in the esterification with diazomethane of a mixture of DNP-glycine and DNP-L-phenylalanine. DNP-L-phenylalanine (4·93 mg.) and DNP-glycine (2·96 mg.) were dissolved in dioxan (4 ml.) and esterified with 10 times the theoretical amount of diazomethane. The reaction mixture was evaporated *in vacuo*, the residue dissolved in benzene, adsorbed on an alumina column (22 × 320 mm.), and the chromatogram developed with benzene:light petroleum, b.p. 60–80° (1:3, v/v). A narrow, very intense red zone was retained at the top of the column, while the rest of the material separated into two bright-yellow zones. These were eluted separately, and after evaporation *in vacuo* each residue was dissolved in ethanol and the amount of material was estimated spectrophotometrically at the 342 mμ. absorption maximum.

Yield of the faster zone $R = 0·185$ (DNP-L-phenylalanine methyl ester) was 5·12 mg. (99·6%), while the yield of the slower zone $R = 0·11$ (DNP-glycine methyl ester) was 3·13 mg. (98·4%).

The '*R* value' is an expression of the relative rate of movement of the adsorption zone (band) to the movement of the solvent.

Stability of the DNP-amino-acid derivatives to acid hydrolysis

Two methods were used for this investigation, both using the same hydrolytic treatment, but each one a different method of recovery of the unchanged substance.

First method. This method was similar to that described by Sanger (1945) and Porter & Sanger (1948). Approximately 0·02% solutions of DNP-amino acids in 6 N-HCl were boiled under reflux for varying periods of time. After evaporation *in vacuo*, the residue was dissolved in water-saturated ether, the solution passed through a 2 g. silica:ether column to remove HCl, and the material was then purified on a 6 g. silica:$CHCl_3$ column.

The fast faint-yellow zone (R 1·4) was neglected, and the only other zone obtained in each case was evaporated *in vacuo*, the residue dissolved in an appropriate volume of $NaHCO_3$ solution (1%, w/v), and estimated spectrophotometrically.

Second method. The DNP-amino acids were subjected to the same conditions of acid hydrolysis as in the first method. After evaporation *in vacuo* the residue was dissolved in dioxan, esterified with diazomethane, the esterification mixture evaporated *in vacuo*, the residue dissolved in $CHCl_3$ and fractionated chromatographically on alumina columns. A small amount of impurity was completely washed out of the column, the remainder was very strongly adsorbed at the top, whereas the required compound occupied the centre portion of the column. It was eluted with acetone, filtered, and the acetone evaporated to dryness *in vacuo* in a weighed flask. The results obtained by both methods are given in Table 2.

Table 2. *Recovery of DNP-amino acids after exposure to hydrolytic conditions as used for the hydrolysis of proteins*

(The unaltered DNP-amino acids were recovered either as free DNP-amino acids by partition chromatography (1st method), or as DNP-amino acid methyl esters by adsorption chromatography (2nd method).)

DNP-amino acid	Hydrolysis method	Duration of hydrolysis (hr.)	Initial weight of material (mg.)	DNP-amino acid recovered (mg.)	Method of recovery	Recovery of DNP-amino acid (%)
Glycine	Reflux with 6 N-HCl	8	5·13	1·40	1st	27·3
Glycine	Reflux with 6 N-HCl	8	5·33	1·39	1st	25·1
Glycylglycine	Reflux with 6 N-HCl	8	4·32	0·960	1st	27·5
Glycine	Reflux with 6 N-HCl	8	92·2	43·0	2nd	42·1
Glycine	100° (*in vacuo*) 6 N-HCl	8	5·14	2·59	1st	50·4
L-Phenylalanine	Reflux with 6 N-HCl	8	3·98	2·87	1st	72·1
L-Phenylalanine	Reflux with 6 N-HCl	8	161·6	128·0	2nd	77·0
ε-DNP-L-Lysine.HCl, H_2O	Reflux with 6 N-HCl	8	6·9	5·37	1st	77·8

The free amino groups of insulin

Crystalline insulin (Boots Pure Drug Co. Ltd.; 22·05 units/mg.; 1·15% NH_2-N (Van Slyke 30 min. reaction period)) was recrystallized following essentially the methods of Romans, Scott & Fisher (1940) and Lens (1948). The product finally obtained had NH_2-N = 1·00% (Van Slyke). (Found: C, 49·1; H, 6·8; N, 15·3; S, 3·1%.) This insulin was treated with FDNB following the procedure of Sanger (1945), when DNP-insulin (Found: C, 47·3; H, 6·1; N, 15·4; S, 2·5%) was obtained as a yellow powder.

DNP-insulin (162·1 mg.) was refluxed with 6N-HCl (25 ml.) for 8 hr. in a glycerol bath (bath temperature 123°). The hydrolysate cooled to room temperature, was extracted six times with ether (15 ml.); after the fourth extraction the ether layers were colourless. The combined ether layers were three times extracted with water (6 ml.); the water layers were colourless after the first extraction. The combined ether layers (the ether-soluble fraction), and the combined water layers (the water-soluble fraction) were separately evaporated *in vacuo*.

The ether-soluble fraction. The yellow residue was dissolved in dioxan (5 ml.) and esterified using approximately 10 times the required amount of diazomethane. The solution was evaporated *in vacuo*, the residue dissolved in benzene (2 ml.), and adsorbed on an alumina column (11 × 250 mm.) prepared with benzene:light petroleum, b.p. 60–80° (1:3, v/v). The rate of flow was 31 ml./hr. After development with 60 ml. of the benzene:light petroleum mixture, an extremely faint buff-coloured zone (*R* 1·2) was washed out of the column and was discarded. The appearance of the chromatogram was (the bracketed figures indicate the width of zones in mm.): top, extremely thin zone, bright-red, (2) orange, (13) white, (14) yellow (*R* 0·035; zone *A*), (30) white, (48) yellow (*R* 0·115; zone *B*), (16) white, (70) yellow (*R* 0·19; zone *C*).

Zone C. The *R* value compares well with the value of 0·185 obtained for synthetic DNP-L-phenylalanine methyl ester under similar conditions of chromatographic analysis. A mixed chromatogram with the synthetic material containing approximately equal amounts of both substances was chromatographically homogeneous.

A sample corresponding to 16·21 mg. of DNP-insulin was concentrated to dryness *in vacuo* and dissolved in 100 ml. of ethanol. The spectrum was identical with that of the synthetic DNP-L-phenylalanine methyl ester. An optical density of 0·276 was observed at the 342 mμ. maximum where $\epsilon = 17\,200$; calculation gives 0·554 mg. of DNP-phenylalanine methyl ester for 16·21 mg. of DNP-insulin. Assuming the validity of the experimentally established value of 72·1% for the recovery of DNP-phenylalanine under the hydrolytic conditions used, and a molecular weight of 14800 for the DNP-insulin submolecule (calculated from sulphur contents), calculation leads to 2·03 *N*-terminal phenylalanine residues per insulin submolecule of mol.wt. 12000.

Zone B. The *R* value of 0·115 found for this zone corresponds very well with the value of 0·11 obtained for synthetic DNP-glycine methyl ester under similar chromatographic conditions. A chromatogram after mixing with the synthetic derivative gave a single band.

A sample corresponding to 16·21 mg. of DNP-insulin concentrated *in vacuo* and dissolved in 25 ml. of ethanol showed an optical density of 0·516 at the 342 mμ. absorption maximum. The spectrum was identical with that of the synthetic derivative. This is equivalent to 0·191 mg. of substance in 16·21 mg. of DNP-insulin. Assuming the correctness of the value of 27·5% for the recovery of DNP-glycine under the hydrolytic conditions used, calculation gives 2·48 *N*-terminal glycine residues per insulin submolecule of mol.wt. 12000.

Zone A. The *R* value of 0·035 found for this zone does not correspond to that of any DNP-amino acid methyl ester as yet investigated. Mixed chromatograms with synthetic derivatives of glycine, phenylalanine, ε- and di-DNP-L-lysine methyl esters show that the substance contained in this band is a chromatographically distinct compound.

A sample corresponding to 15·20 mg. of DNP-insulin was concentrated *in vacuo*, dissolved in 25 ml. of ethanol and the ultraviolet-spectrum of the solution was examined. The spectrum differed slightly from those of the derivatives of glycine, phenylalanine and lysine. Attempts were made to weigh the residue from this zone, but no significance could be attached to the result owing to the extreme difficulty of completely removing the solvent.

A rough calculation can be made of the number of moles of material present in this zone if one assumes that the substance has at its 338 mμ. maximum a molecular extinction coefficient (17200) similar to the derivatives of glycine and phenylalanine at their 342 mμ. absorption maxima. If the further assumption is made that the compound has a molecular weight of about 300, then the amount of material derived from 15·2 mg. of DNP-insulin would be 0·135 mg. This compares with 0·179 mg. of DNP-glycine methyl ester, and with 0·520 mg. of DNP-phenylalanine methyl ester present in the same amount of DNP-insulin. This necessarily very rough calculation was made in order to give some idea of the relative amount of material present in the unidentified zone.

The water-soluble fraction. The residue from the water-soluble fraction of the DNP-insulin hydrolysate was rehydrolysed as before for another 8 hr., concentrated *in vacuo*, and the residue esterified in the same way as described for the esterification of ε-DNP-L-lysine.HCl, H_2O. After evaporation of the esterification mixture *in vacuo* at room temperature, one-fifth of the residue dissolved in a 5% solution of methanol in $CHCl_3$ (2 ml.) was adsorbed on an alumina column (10 × 267 mm.), prepared with benzene and developed with 2% methanol in benzene. The appearance of the chromatogram was identical with that described for the methyl ester of ε-DNP-L-lysine.

The *R* value (0·14) of the main zone was in fair agreement with the *R* value of 0·10 obtained for the main zone in the esterification of synthetic ε-DNP-L-lysine HCl, H_2O under similar conditions. Mixed chromatograms showed the chromatographic homogeneity with synthetic ε-DNP-L-lysine methyl ester.

A sample corresponding to 9·19 mg. of DNP-insulin dissolved in 50 ml. of ethanol had a spectrum identical with that of ε-DNP-L-lysine methyl ester hydrochloride, and an optical density of 0·249 at the 348 mμ. absorption maximum corresponding to 0·271 mg. of substance. A correction factor for the loss through decomposition during concentration *in vacuo* followed by rechromatography corrects the value to 0·286 mg. of ε-DNP-lysine methyl ester hydrochloride for 9·19 mg. of DNP-insulin. Assuming the correctness of the value obtained for the amount of decomposition of the substance under the hydrolytic conditions, calculation gives 2·10 free ε-amino-groups of lysine per insulin submolecule of mol.wt. 12000.

DISCUSSION

The examination of the *N*-terminal amino acids carrying the free amino groups in insulin led Sanger (1945) to the conclusion that in each insulin submolecule of molecular weight 12000 there are two *N*-terminal residues of glycine, two of phenylalanine, and two lysine residues carrying free ϵ-amino groups. Sanger concluded that the insulin submolecule was made up of four open peptide chains, two of these having terminal glycyl residues, and the other two having terminal phenylalanyl residues. These conclusions are based on the identification and estimation by partition-chromatographic methods of the DNP-amino acids in the hydrolysate of DNP-insulin. Owing to the instability of DNP-amino acids under the hydrolytic conditions used for the preparation of the hydrolysate, correction factors for the decomposition of the DNP-amino acids during hydrolysis had to be used in those calculations.

In the present investigation an adsorption-chromatographic method for the separation and estimation of methyl esters of DNP-amino acids is described. An application of this method to the study of the free amino groups of insulin led to the following results. In the insulin submolecule of molecular weight 12000 there are two *N*-terminal phenylalanyl residues, but the amount of DNP-glycine found may represent 2 or 3 (2·48) glycyl residues with equal probability. In addition, an unidentified substance was found; a rough calculation of the amount present indicates an order of magnitude similar to that of the amount of DNP-glycine.

It is interesting to note that in experiments with the same DNP-insulin, carried out in a way similar to that used by Sanger (1945), the unidentified substance was not observed. The explanation might be that in separations by partition chromatography the DNP derivative in question formed so wide a zone that the diffuse colour could not be observed.

As the calculation of the number of *N*-terminal residues depends on the amount of decomposition of DNP-amino acids during the hydrolysis of the DNP-protein, we have carefully re-examined the magnitude of those correction factors. Even by standardizing most carefully the hydrolytic conditions the recovery of the unchanged DNP-amino acids varies to a considerable extent. For instance, in the case of DNP-glycine the recoveries vary between 25 and 42% depending on the method used for the estimation of the yield of the unchanged product after the hydrolytic treatment. But there is in this connexion an additional complication. The rate of decomposition of any DNP-amino acid may be determined by the presence of various products of protein hydrolysis. Porter & Sanger (1948) reported that there is no change in the stability of DNP-amino acids in the presence of added globin. Desnuelle, Rovery & Fabre (1951), however, report a considerable effect of the presence of various proteins on the recovery of DNP-aspartic acid. For all these reasons it seems to be quite uncertain how far the results of calculations using the above-mentioned correction factors really correspond to the amounts of the *N*-terminal amino acid residues actually contained in insulin.

In this preliminary investigation only small amounts of insulin were used, and for this reason an attempt was not made to identify the chromatographically isolated derivatives by their melting points and by other physico-chemical methods. The provisional identification of the isolated substances was made by determinations of the *R* values under standard conditions, and by mixing each isolated substance with pure authentic specimens and ascertaining the chromatographic homogeneity of the mixtures.

SUMMARY

1. Methyl esters of *N*-2:4-dinitrophenyl (DNP) derivatives of a number of amino acids have been prepared.

2. The separation of mixtures of these derivatives by adsorption chromatography on alumina columns was investigated.

3. The stability to acid hydrolysis of some DNP-amino acids was investigated by two different methods.

4. The new method of separation of mixtures of DNP-amino acids (by adsorption chromatography of their methyl esters) was applied to the re-investigation of the free amino groups of insulin.

5. Assuming a molecular weight of 12000 it is shown that two free amino groups are on phenylalanine residues, two or three on glycine residues, and two represent the ϵ-amino groups of lysine. In addition, a new, unidentified substance bearing free amino groups was found.

6. It is suggested that owing to the difficulty of an exact assessment of the magnitude of correction factors for the decomposition of DNP-amino acids during protein hydrolysis, it must be considered as quite uncertain how far the calculated values for the amount of *N*-terminal amino acid residues really correspond to the amount actually contained in insulin.

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Studies on the Determination of the Sequence of Amino Acids in Peptides and Proteins

3. THE SYNTHESIS OF 3-(4′-DIMETHYLAMINO-3:′5′-DINITROPHENYL)HYDANTOIN DERIVATIVES OF VARIOUS AMINO ACIDS, AND THEIR USE FOR THE DETERMINATION OF *N*-TERMINAL AMINO ACIDS

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The reaction of phenyl *iso*cyanate with amino acids has been known for many years. Some phenylcarbamyl derivatives obtained in such condensations were described by Paal (1894), and Raper (1907) condensed various fractions of peptones obtained from serum albumin with the same reagent. Bergmann, Miekeley & Kann (1927) condensed dipeptides with phenyl *iso*cyanate, and hydrolysed the products to give hydantoins corresponding to the amino acids which were originally the *N*-terminal residues. Abderhalden & Brockmann (1930) showed that the hydantoins are split from the peptide chain under mild hydrolytic conditions (treatment for 30 min. at 60–65° with methanol saturated with HCl), and that under these conditions the residual peptide was hydrolysed to a small extent only. Edman (1950) found that reaction with phenyl *iso*thiocyanate gives phenylthiocarbamyl peptides, from which under even milder hydrolytic conditions (nitromethane saturated with hydrochloric acid) than those used by Abderhalden & Brockmann (1930) the *N*-terminal amino-acid residue splits off as a hydantoin. Hopkins & Wormall (1934) have shown that phenyl *iso*cyanate reacts with the *N*-terminal amino acids of insulin, and Jensen & Evans (1935) were able to isolate the phenylhydantoin of phenylalanine from the hydrolysate, thus demonstrating for the first time the occurrence of phenylalanine as an *N*-terminal residue in insulin.

In order to facilitate the isolation in a pure state of the individual hydantoins from mixtures representing several *N*-terminal residues, and to allow quantitative determinations to be carried out, we have synthesized a coloured *iso*cyanate, the 4-dimethylamino-3:5-dinitrophenyl *iso*cyanate. The introduction of the chromophoric groups into the benzene nucleus has also the effect of improving the adsorption characteristics of the hydantoins, and a method of chromatographic separation of their mixtures was found.

The names of the substances described in this paper, when in accordance with the agreed chemical nomenclature, are rather long. We have used abbreviated names as follows: 4-dimethylamino-3:5-dinitrophenyl *iso*cyanate is called DDPI, and reaction products of this with the amino groups of amino acids are called DDP-ureidoamino acids, e.g., DDP-ureidoglycine. The corresponding hydantoin is called glycine-DDP-hydantoin.

EXPERIMENTAL AND RESULTS

Preparation of 4-*dimethylamino-*3:5-*dinitrophenyl* iso*cyanate (DDPI)*

4-Dimethylamino-3:5-dinitrobenzoic acid was prepared by a slightly modified method of Ullmann (1909). To a solution of 4-chloro-3:5-dinitrobenzoic acid (10·8 g.) in water (150 ml.) containing K_2CO_3 (6·3 g.) was added a solution of dimethylamine (33%, w/w; 7·5 ml.). After heating the solution on a steam bath for 15 min., and cooling to room temperature, HCl (sp.gr. 1·18; 15 ml.) was added slowly while stirring, when a thick, yellow precipitate appeared, which was filtered off, washed with water and dried. Yield 10·8 g., m.p. 226–236°; recrystallized from ethanol (60 ml.), yield 8·2 g., m.p. 244–246° (decomp.) (75%; Ullmann, 1909, gives m.p. 246°).

This substance (17·3 g.) was refluxed in dry benzene (150 ml.), while $SOCl_2$ (14 ml.) dissolved in benzene (75 ml.) was slowly added. The mixture was then refluxed for 2 hr., the residue evaporated *in vacuo*, dissolved in dry benzene

(50 ml.), again evaporated and the residue dried overnight in a vacuum desiccator containing pellets of KOH. Yield 18·4 g. (99%), m.p. 117–118°. Recrystallization from light petroleum b.p. 80–100° (800 ml.) yielded 17·0 g. of crystals of *4-dimethylamino-3:5-dinitrobenzoyl chloride*, m.p. 120–121°. (Found: C, 39·2; H, 2·9; N, 15·6; Cl, 13·2. Calc. for $C_9H_6N_3O_5Cl$ requires C, 39·5; H, 2·9; N, 15·4; Cl, 13·0%.)

The acid chloride (5·47 g.) was dissolved in acetone (80 ml.), the solution cooled to 0° and, whilst stirring, NaN_3 (1·6 g.) dissolved in water (8 ml.) was added in a continuous stream during 10 min. The stirring was continued for a further 15 min., when water (150 ml.) was added quickly yet dropwise, and the stirring was continued for another 15 min. at 0°. The precipitate was filtered off, washed with water and dried. The yield of *4-dimethylamino-3:5-dinitrobenzoyl azide* was 5·43 g. (97%), m.p. 132–133° (decomp.) (Found: C, 38·6; H, 2·9; N, 27·2. $C_9H_6O_5N_6$ requires C, 38·6; H, 2·9; N, 30·0%.) Analysis was unsatisfactory owing to violent decomposition when heated.

The acid azide (10 g.) was dissolved in dry toluene (250 ml.) at room temperature in a flask fitted with a mercury-seal stirrer, a reflux condenser closed with a $CaCl_2$ tube which was connected with a cold trap (cooled in solid CO_2) connected with a nitrometer charged with a KOH solution (30:100, w/v). A stream of dry CO_2 was passed through the apparatus for 30 min. with the nitrometer disconnected. The nitrometer was then connected, and the passage of CO_2 continued until only microbubbles reached the top of the KOH solution (rate of gas collection was then 1 ml. in 5 min.). The passage of CO_2 was then stopped. The flask containing the azide solution was immersed into a glycerol bath, and the temperature was cautiously raised until there was a brisk evolution of gas round the stirrer (80°). The temperature of the reaction mixture was then slowly raised to 95°, and maintained at that temperature until the evolution of gas ceased (about 2 hr. 30 min.; nitrometer reading 30 ml.). The CO_2 was then passed again until the rate of gas collection was again 1 ml. in 5 min. (a further 733 ml. of N_2 was measured). The total gas volume at s.t.p. was 808 ml. (Theory, 800 ml.). The solution was left overnight at room temperature, evaporated *in vacuo* (bath temp. <45°), and the residue dried over H_2SO_4. Yield, 9·5 g., m.p. 96–100°. After two recrystallizations from light petroleum (b.p. 60–80°) orange-red plates of *4-dimethylamino-3:5-dinitrophenyl isocyanate* were obtained; yield 8·5 g. (95%), m.p. 101–102°. (Found: C, 42·6; H, 3·2; N, 22·1. $C_9H_5O_5N_4$ requires C, 42·8; H, 3·2; N, 22·2%.) The substance was readily soluble in acetone and dioxan, soluble in $CHCl_3$ and benzene, and sparingly soluble in ether and high-boiling light petroleum. The substance is not wetted by cold water but reacts on boiling.

Reaction of DDPI with aniline

A solution of aniline in benzene (1:10, w/w; 1 ml.) was added to a solution of DDP *iso*cyanate (250 mg.) in dry benzene (2·5 ml.). A solid mass of crystals formed immediately. The reaction was markedly exothermic. Benzene (50 ml.) was added, the solution warmed and the product allowed to crystallize. The crystals (m.p. 190–191°) were recrystallized from benzene when *N-(4-dimethylamino-3:5-dinitrophenyl)-N'-phenyl urea*, m.p. 190–191°, was obtained. (Found: C, 52·4; H, 4·4; N, 20·2. $C_{15}H_{13}O_5N_5$ requires C, 52·2; H, 4·4; N, 20·3%.) The same substituted diphenyl urea was obtained by refluxing for 5 hr. 4-dimethylamino-3:5-dinitrobenzoyl azide with aniline dissolved in dry benzene; m.p. 189–190°, mixed m.p. 189–191°.

Reaction of DDPI with water

Owing to its insolubility DDP *iso*cyanate reacts with water only very slowly. However, under the conditions of the condensation with the free α-amino groups of amino acids and peptides, where the *iso*cyanate is first dissolved in a solvent miscible with water, a much faster reaction was expected.

DDPI (500 mg.) was dissolved in acetone (5 ml.) and added dropwise to a Na_2CO_3 solution (2·5:100, w/v; 10 ml.) stirred at 0°. The stirring was continued for 1 hr., the dark-red precipitate filtered off, washed with water and dried *in vacuo*. Yield, 320 mg.; m.p. 215–217° (decomp.). Recrystallized from benzene, deep-red crystals of *bis-(4-dimethylamino-3:5-dinitrophenyl)urea* (*di-DDP-urea*) were obtained; m.p. 247° (decomp.). (Found N, 23·5. $C_{17}H_{18}O_9N_8$ requires N, 23·4%.)

The filtrate from the reaction mixture was evaporated *in vacuo*, the residue dissolved in hot water (20 ml.) and left at 0° overnight. The bright-red crystals were filtered off and washed with ice-water. Yield 100 mg.; m.p. 154–156°; recrystallization from CCl_4 yielded *4-dimethylamino-3:5-dinitrophenylamine*, m.p. 156–157°. (Found: C, 42·5; H, 4·7; N, 24·7. $C_8H_{10}N_4O_4$ requires C, 42·5; H, 4·4; N, 24·8%.) A small sample was dissolved in ether, and to this solution was added an ether solution of a roughly equivalent quantity of DDPI. When the solution was concentrated to a small volume dark-red crystals of di-DDP-urea were obtained.

Reaction of DDPI with esters of amino acids and peptides

A solution of DDPI (1·25 g.; 5·0 mmoles) in $CHCl_3$ (5 ml.) was shaken at 0° with a solution of glycine ethyl ester (0·51 g.; 5·0 mmoles) in $CHCl_3$ (2 ml.). After standing overnight at 0° the product was precipitated with light petroleum b.p. 60–80° (30 ml.), the red, oily precipitate solidified on rubbing, was allowed to stand at 0° for 1 hr., and was then filtered off and recrystallized first from ethanol and then from ethanol:water (50:50, v/v); yield 1·5 g. (85%) of *4-dimethylamino-3:5-dinitrophenylcarbamylglycine ethyl ester* (*DDP-ureidoglycine ethyl ester*), m.p. 177–178°. (Found: C, 44·0; H, 4·8; N, 19·7. $C_{13}H_{17}N_5O_7$ requires. C, 44·0; H, 4·8; N, 19·7%.)

In a similar way glycylglycine ethyl ester gave *DDP-ureidoglycylglycine ethyl ester* (yield 78%), m.p. 184–185°. (Found: C, 43·8; H, 4·7; N, 20·1. $C_{15}H_{20}O_8N_6$ requires C, 43·7; H, 4·9; N, 20·4%.)

DL-Alanylglycylglycine (530 mg.; 2·3 mmoles) was suspended in ethanol (15 ml.) previously saturated with dry HCl. The suspension was warmed on a steam bath for 5 min. until the solid was completely in solution, and then cooled quickly to room temperature. The solution was evaporated *in vacuo*, the residue dissolved in ethanol and precipitated with light petroleum (b.p. 60–80°) several times. The precipitate was then dissolved in water (1 ml.), $CHCl_3$ (5 ml.) was added, and the mixture cooled to −15°; K_2CO_3 was added in small quantities with constant agitation until the aqueous layer became a thick slurry. The $CHCl_3$ extract was decanted, the slurry thoroughly extracted with small portions of $CHCl_3$, and the combined $CHCl_3$ extracts were

shaken for 1 hr. with CaO, filtered and the filtrate cooled to −15°. DDPI (650 mg.; 2·6 mmoles) dissolved in $CHCl_3$ (10 ml.) and cooled to −15° was then mixed with the above extract; after standing for 2 hr. at 0°, the solution was evaporated *in vacuo*, ether (10 ml.) was added, and the oil when rubbed solidified almost immediately; it was filtered off, washed with ether and light petroleum (b.p. 40–60°), and three times crystallized from ethanol:water (50:50); yield, 560 mg. (45%) of orange crystals of *DDP-ureido-*DL*-alanylglycylglycine ethyl ester*, m.p. 195° (decomp.). (Found: C, 44·8; H, 5·05; N, 20·4. $C_{18}H_{25}O_9N_7$ requires C, 44·8; H, 5·2; N, 20·3%.)

Saponification of the ethyl esters. DDP-ureidoglycine ethyl ester (100 mg.) suspended in 0·1 N-NaOH (5 ml.) was shaken at room temperature for 30 min. when the crystals dissolved. The solution was filtered, acidified with 2 N-HCl, and the mixture kept for 2 hr. at 0°. The precipitate was filtered off and dissolved in ethanol (0·5 ml.); water was added (2 ml.), the mixture dissolved by warming and allowed to cool slowly; the deep-red crystals of *4-dimethylamino-3:5-dinitrophenylcarbamylglycine* (*DDP-ureidoglycine*) were filtered off; yield 65 mg.; m.p. 178° (decomp.). (Found: C, 40·4; H, 4·0; N, 21·1. $C_{11}H_{13}N_5O_7$ requires C, 40·4; H, 4·0; N, 21·4%.) The mother liquors were extracted with ethyl acetate, the solution evaporated *in vacuo*, and the residue recrystallized from water; yield 15 mg.; m.p. 175° (decomp.). The total yield was 92%.

In a similar way DDP-ureidoglycylglycine, m.p. 130–136° (decomp.), and DDP-ureidoalanylglycylglycine, m.p. 140–145° (decomp.), were obtained. The substances retain small amounts of solvents which were difficult to eliminate, and for this reason were not obtained in an analytically pure state.

Reaction of DDPI with amino acids and peptides

Preparation of DDP-ureidoglycine. Glycine (300 mg.; 4·0 mmoles) was dissolved in dioxan:water (50:50, v/v; 20 ml.) and the solution was brought to pH 9·6 (glass electrode) by addition of 0·1 N-NaOH. The temperature was brought to 0°, and DDPI (1·1 g.; 4·4 mmoles) dissolved in dioxan (10 ml.) was added dropwise with constant stirring. By addition of 0·1 N-NaOH the 'pH' was maintained at 9·6 during the 30 min. required for the addition of the DDPI solution. The stirring was prolonged for another 30 min., during which time the pH reading did not alter appreciably. The solution was brought to 'pH 6', and evaporated *in vacuo*. The residue was taken up in water, the pH adjusted to 9·5 and any DDP-amine present was removed by extraction with ether. An equal volume of dioxan was then added and the solution treated in the same way as before with a solution of DDPI (0·5 g.) in dioxan (5 ml.). After the reaction was completed the solution was acidified with HCl, left overnight at 0°, and the precipitate was filtered off; yield, 1·3 g. (100%) of red crystals, m.p. 160–170° (decomp.). Recrystallization from boiling water gave 1·2 g. of 4-dimethylamino-3:5-dinitrophenylcarbamylglycine (DDP-ureidoglycine), m.p. 176°. This product (138·4 mg.) titrated with 0·0998 N-NaOH required 4·20 ml. (99%).

Preparation of DDP-ureidoglycylglycine. Glycylglycine hydrochloride monohydrate (95 mg., 0·51 mmole) was dissolved in water, the pH was adjusted to 10 and dioxan (10 ml.) was added. The solution was treated with a solution of DDPI (251 mg., 1 mmole) in dioxan (5 ml.) in the manner described for the preparation of DDP-ureidoglycine. On completion of the addition the solution was stirred for 15 min., and then more DDPI (80 mg., 0·32 mmole) dissolved in dioxan was added in the same way. The reaction mixture was then adjusted to pH 4·5, the precipitate filtered and washed free of water-soluble red components. Yield 130 mg.; m.p. 225° (decomp.). The filtrate was evaporated *in vacuo*, the residue extracted with a $NaHCO_3$ solution (5:95, w/v), filtered, and the insoluble residue was washed on the filter with the $NaHCO_3$ solution until no more colour was extracted. The yield of the precipitate was 44 mg. m.p. 230°. The filtrate and washings were acidified with HCl, and the mixture left overnight at 0°. The precipitate was filtered off and washed with ice-water; yield 190 mg. (98%) m.p. 125–130° (decomp.). Recrystallization from ethanol:water mixtures failed to give a crystalline product. The m.p. remained at 130–136° (decomp.), the substance retaining small amounts of solvent which could not be eliminated. The mixed m.p. with the DDP-ureidoglycylglycine prepared from the ethyl ester was unchanged.

This substance (28·7 mg.), dissolved in dioxan:water (50:50, v/v; 8 ml.), was titrated (glass electrode) with 0·0530 N-NaOH; it required 1·39 ml. (98·5%).

*Preparation of DDP-ureido-*DL*-alanylglycylglycine.* DL-Alanylglycylglycine (104 mg.) was treated with a solution of DDPI in the same way as described for glycylglycine. After the reaction was completed, the reaction mixture was concentrated to remove most of the dioxan, acidified and extracted twice with ethyl acetate. The ethyl acetate solution was extracted several times with aqueous $NaHCO_3$ (5:95, w/v) until no more coloured substance could be extracted. The $NaHCO_3$ solution was acidified, extracted with ethyl acetate and the ethyl acetate solution evaporated *in vacuo*. The residue was dissolved in dry acetone, filtered, and the acetone solution evaporated *in vacuo*. The residue was rubbed in ether and yielded 228 mg. of DDP-ureido-DL-alanylglycylglycine (98%), m.p. 140–145° (decomp.). The mixed m.p. with the DDP-ureido-DL-alanylglycylglycine prepared from the ethyl ester was unchanged.

This substance (16·3 mg.) dissolved in dioxan:water (50:50, v/v) and titrated with 0·0265 N-NaOH required 1·34 ml. (99%).

*Preparation of DDP-ureido-*L*-tryptophan.* L-Tryptophan (103 mg.) was dissolved in a mixture of 0·1 N-NaOH (5 ml.) and dioxan (5 ml.). DDPI (255 mg.) was added in the manner described above. The residue of di-DDP-urea, when the acid had been extracted with the $NaHCO_3$ solution, was 122 mg. The bicarbonate extract after washing with ether yielded on acidification 225 mg. (98%) of a very finely divided precipitate which was separated by centrifugation. The substance was dissolved in methanol:water (50:50) and a slow evaporation of the solution at 20° yielded well formed microscopic crystals of *4-dimethylamino-3:5-dinitrophenylcarbamyl-*L*-tryptophan* (*DDP-ureido-*L*-tryptophan*), m.p. 155° (decomp.). The m.p. was not very sharp, the crystals were apparently impure and softened at 135°. Analysis was not entirely satisfactory. (Found: C, 52·1; H, 5·0; N, 18·1. $C_{20}H_{20}O_7N_6$ requires C, 52·7; H, 4·4; N, 18·4%.)

Preparation of the N^3*-(4-dimethylamino-3:5-dinitrophenyl)-substituted hydantoins of various amino acids* (*amino acid-DDP-hydantoins*)

The DDP-hydantoins of six amino acids were prepared. As an example, the preparation of 3-(4′-dimethylamino-3′:5′-dinitrophenyl)-5-*iso*propylhydantoin (valine-DDP-hydantoin) will be described.

Table 1. *DDP-hydantoins prepared from various amino acids by the method given in the text*

Derived from amino acid	M.p. (°)*	Analysis: Found (%) C	Found (%) H	Found (%) N	Requires (%) C	Requires (%) H	Requires (%) N
Glycine	213–214	42·9	3·8	22·6	42·7	3·6	22·6
DL-Alanine	174–175	44·5	4·1	21·8	44·6	4·05	21·7
DL-Phenylalanine	173–174	54·2	4·5	17·8	54·1	4·3	17·55
DL-Valine	163–164	47·8	4·1	19·8	47·9	4·34	19·9
L-Tyrosine	200	52·6	4·0	17·0	52·1	4·1	16·9
DL-Serine	177	42·1	3·8	20·9	42·5	3·9	20·6

* All melting points are corrected.

DL-Valine was treated with DDPI as described above for the preparation of DDP-ureido-amino acids. After the reaction the solution was concentrated *in vacuo*, extracted completely into ethyl acetate, and the amino acid derivative extracted from the ethyl acetate with aqueous $NaHCO_3$ (5:95, w/v). The bicarbonate extract was washed twice with ether, acidified, and evaporated *in vacuo*. The residue dissolved in acetone was filtered, the filtrate evaporated *in vacuo*, and the residue rubbed with light petroleum, b.p. 40–60°. The yellow powder was dissolved in a very small amount of ethanol, N-HCl (10 vol.) was added, and the solution refluxed for 3 hr. Orange crystals formed gradually. After standing at 0° overnight, the substance was filtered off and recrystallized from ethanol:water (50:50, v/v). The data on the *hydantoins* prepared in a similar way are given in Table 1.

It is clear that DDPI has not reacted with the hydroxyl groups of tyrosine and serine.

It was found that, at least in the case of the DDP-ureido-amino acids which we have prepared, ring closure leading to the hydantoins can most conveniently be effected by refluxing with 2N-HCl for 1–2 hr. Under these conditions the hydantoins were perfectly stable and were recovered in 98–100% yields. Refluxing with 6N-HCl leads to decomposition and poor yields. It is probable that the decomposition occurs before ring closure. In the case of DDP-ureido-amino acid esters and in DDP-ureido peptides where ring closure proceeds more easily than in the case of DDP-ureido-amino acids, the refluxing with 6N-HCl gives good yields of hydantoins.

DDP-ureidoglycine (8·5 mg.) was suspended in 2N-HCl (2 ml.) and refluxed for 2 hr. The red crystals went completely into solution. After 1 hr. yellow crystals precipitated. The mixture was evaporated *in vacuo*, the residue washed with ethanol and evaporated 4 times to remove HCl and moisture. The residue was dissolved in acetone (1 ml.), benzene (9 ml.) was added, and the solution was adsorbed on an alumina column (see below). After development with benzene:ethanol (99·75:0·25, v/v), the main zone was eluted and estimated colorimetrically. The yield was 99% of theoretical value. The solution was evaporated *in vacuo*, and the m.p. of the residue, and the mixed m.p. with an analytically pure specimen of glycine-DDP-hydantoin were practically the same (208–210°).

DDP-ureidoglycylglycine (9·8 mg.) was refluxed with 2N-HCl (2 ml.) for 2 hr. The chromatographic purification was carried out as in the experiment above. On the alumina column crystals of glycine hydrochloride could be seen on the top of the column. The main zone eluted and estimated colorimetrically gave a yield of 98·5% of the theoretical yield of glycine-DDP-hydantoin. The m.p. and the mixed m.p. was 207–210°.

It was found that hydantoin formation proceeds more easily from DDP-ureido-amino acid esters than from DDP-ureido-amino acids. When, for instance, DDP-ureidoglycine was refluxed with 6N-HCl for 1 hr. the yield of hydantoin was very poor, while DDP-ureidoglycine ethyl ester treated in the same way gave a 92% yield of the hydantoin.

A similar observation was made for DDP-ureido-amino acids contained in peptides. DDP-ureidoglycine kept for 4 days at 40° with nitromethane saturated with HCl gave a 48% yield of the hydantoin, while DDP-ureidoglycylglycine under the same conditions gave a 78% yield. It was found that DDP-ureidoglycylglycine kept for 5–6 days at 40° under the same conditions gave a practically quantitative yield of glycine-DDP-hydantoin.

Stepwise degradation of DL-*alanylglycylglycine*

DDP-ureido-DL-alanylglycylglycine (36·4 mg.) was mixed with nitromethane previously saturated with dry HCl (10 ml.) and was left standing for 5 days at 40°. After evaporation *in vacuo* (<30°) the residue was washed several times with ethanol and evaporated to dryness after each washing to remove HCl. The residue was then extracted with several small portions of dry acetone and the extract filtered. The filter was washed to dissolve all traces of colour. The filter containing the glycylglycine hydrochloride was kept for further investigation.

The acetone solution was evaporated *in vacuo*, the residue dissolved in as small a volume of acetone as possible, and the solution was diluted with benzene (10 vol.). This solution was then adsorbed on an alumina column, and the chromatogram was developed with benzene:ethanol (99·75:0·25, v/v). There was a thin zone at the top of the column, and a slight fast zone besides the main zone containing the hydantoin. The main zone was eluted, made up to 100 ml. with benzene:ethanol (99·75:0·25, v/v) and estimated colorimetrically. The yield was 99·4% of the theoretical amount of alanine-DDP-hydantoin. The solution was evaporated *in vacuo*, and the residue was hydrolysed by refluxing with a saturated solution of $Ba(OH)_2$ (2 ml.; saturated at room temperature) for 3 hr. The presence of alanine in the hydrolysate was confirmed by paper chromatography (see below).

The residue of the extraction with acetone which remained in the reaction flask and on the filter was taken up with water (7 ml.) followed by dioxan (7 ml.). The solution

was brought to 'pH 9·6' and treated with a DDPI solution in the usual way. The DDP derivative which was obtained (presumed to be DDP-ureidoglycylglycine) was treated with the nitromethane-HCl solution for 6 days at 40°, and the experiment was continued in the same way as described above. The acetone extract yielded 108% of the theoretical amount of glycine-DDP-hydantoin (from the amount expected if the substance had been pure DDP-ureidoglycylglycine). On hydrolysis of the glycine-DDP-hydantoin with $Ba(OH)_2$ only the presence of glycine was detected by paper chromatography.

The residue of the extraction with acetone which remained in the reaction flask and on the filter (presumed to be glycine hydrochloride) was dissolved in water and estimated by formol titration with a glass electrode. A yield of 79% of the theoretical yield of glycine was found. A paper chromatogram showed the presence of glycine.

Colorimetric estimation of the amino acid-DDP-hydantoins

A series of concentrations of standard solutions of various hydantoins were made up in benzene:ethanol (99·75:0·25, v/v) and their absorption was measured with an 'Evans Electroselenium' photoelectric absorptiometer using an Evans dark-blue filter no. 622. Fig. 1 is a graphical representation of the results of these measurements. It can be seen that Beer's Law is obeyed.

Chromatographic separation of some amino acid-DDP-hydantoins

Alumina was prepared by the method already described (Flowers & Reith, 1953). As an example of a chromatographic separation, the resolution of a mixture of the DDP-hydantoins of glycine (2·14 μmoles), DL-alanine (3·02 μmoles), DL-phenylalanine (0·90 μmole), DL-serine (1·93 μmoles), DL-valine (2·62 μmoles) and L-tyrosine (1·39 μmoles) dissolved in benzene:ethanol (95:5, v/v; 1·92 ml.) is described.

The solution was first diluted with light petroleum, b.p. 60–80° (10 ml.), and was then adsorbed on an alumina column (9 × 530 mm.) prepared with a slurry with light petroleum, b.p. 60–80°. After the adsorption on to the column was completed, the ethanol-containing solvent was washed with light petroleum (10 ml.), and the column was developed first with light petroleum:ethanol (99·75:0·25, v/v; 150 ml.) when a zone containing valine-DDP-hydantoin (2·59 μmoles) was recovered in the effluent. Continued development with another 20 ml. of the same solvent brought the zone containing the phenylalanine-DDP-hydantoin (0·90 μmole) into the effluent. On further development with 95 ml. of the same solvent the zone which contained the alanine-DDP-hydantoin (3·04 μmoles) was recovered in the effluent. The development was continued first with light petroleum:ethanol (99:1, v/v; 50 ml.) when a zone containing glycine-DDP-hydantoin (2·09 μmoles) was recovered from the effluent. Further development first with the same solvent (100 ml.), and then with light petroleum:ethanol (95:5, v/v; 43 ml.) brought a zone containing tyrosine-DDP-hydantoin (1·38 μmoles) into the effluent. The final zone which contained serine-DDP-hydantoin (1·97 μmoles) was eluted from the alumina with methanol.

After the colorimetric estimations, the solutions containing the various zones were separately evaporated *in vacuo*, and hydrolysed with a $Ba(OH)_2$ solution (saturated at room temperature). In each of the hydrolysates the amino acid was identified by paper chromatography.

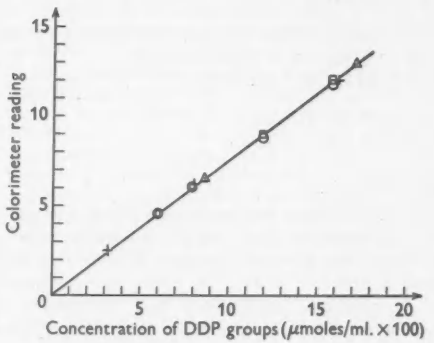

Fig. 1. Absorptiometric measurements of standard solutions of the DDP-hydantoins of glycine (□), alanine (○), valine (+) and serine (△) in benzene-ethanol (99·75:0·25, v/v) solution.

Hydrolysis of the DDP-hydantoins to amino acids

The DDP-hydantoins were refluxed with the saturated $Ba(OH)_2$ solution for 3 hr. Excess $Ba(OH)_2$ was removed by treatment of the solution with CO_2 and filtering, and the DDP-amine and other coloured products were removed by adsorption on a short column of activated alumina (Hopkins and Williams Ltd.). The aqueous solution was evaporated, and the amino acid contained in the residue was identified by paper chromatography with butanol:acetic acid:water (5:1:4, v/v).

DISCUSSION

It was found that the reaction of DDPI with amino acids and peptides proceeds under mild conditions at a pH near to neutrality. The yields were excellent in all cases so far examined. The DDP-ureido-amino acids are converted into DDP-hydantoins by heating with acid. This conversion proceeds more readily in the case of DDP-ureidopeptides so that a method has been developed which permits ring closure with only slight hydrolysis of the residual peptide. During this process the *N*-terminal amino acid of the peptide chain appears as a free DDP-hydantoin in a quantitative yield, and these hydantoins are deeply coloured compounds suitable for chromatography on alumina; the residual peptide can be recovered in good, though not in quantitative yield because of the slight hydrolysis of peptide bonds.

Thus the experimental conditions under which the cyclization is completed are not as yet quite satisfactory for the use of DDP derivatives for sequence determinations as a continuous stepwise degradation procedure. However, the method was found to be excellent for the determination of the *N*-terminal amino-acid residue in a peptide. The

DDP-substituted *N*-terminal amino acid splits off with such ease that mild hydrolytic conditions may be used under which the hydantoins are perfectly stable. Furthermore, as under these conditions the remaining peptide bonds are split only to a very small extent, a possible catalytic effect of the degradation products of certain amino acids on the stability of the marked *N*-terminal amino acids is eliminated.

Mixtures of the deeply coloured DDP-hydantoins can easily be resolved by adsorption chromatography; the isolated DDP-hydantoins can be identified not only by their chromatographic behaviour and their melting point but also by alkaline hydrolysis to regenerate the free amino acid. Paper chromatography can then provide an additional check of identity.

DDPI is thus an excellent reagent for the determination of the *N*-terminal amino acid residue in a peptide.

SUMMARY

1. A red-coloured *iso*cyanate, the 4-dimethylamino-3:5-dinitrophenyl *iso*cyanate (briefly called DDPI) was synthesized.

2. It was found that this *iso*cyanate reacts quantitatively with the free amino groups of amino acids or peptides dissolved in water-dioxan at the apparent pH 9·6 at 0°, giving deep-orange coloured carbamyl derivatives (briefly called DDP-ureido-amino acids or DDP-ureido peptides, for example, DDP-ureido glycine).

3. DDP-ureido-amino acids when refluxed for 2 hr. in 2N-hydrochloric acid, or when treated for several days at 40° with nitromethane saturated with hydrochloric acid, give by ring closure and elimination of water the corresponding hydantoins (briefly called amino acid-DDP-hydantoins, for example, glycine-DDP-hydantoin) in quantitative yields.

4. When DDP-ureido peptides are treated in the same way, the *N*-terminal amino acid carrying the DDP radical splits up, forming by ring closure the corresponding hydantoin which can be recovered in quantitative yields.

5. The amino acid-DDP-hydantoins are deep-orange coloured substances which crystallize well and have sharp melting points. They can be adsorbed on alumina columns, and their mixtures can be conveniently resolved by adsorption chromatography.

6. The scope for the use of these derivatives for the determination of *N*-terminal amino acids in peptides is discussed.

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Studies on the Determination of the Sequence of Amino Acids in Peptides and Proteins

4. THE SYNTHESIS OF 3-(4′-DIMETHYLAMINO-3′:5′-DINITROPHENYL)-2-THIOHYDANTOIN DERIVATIVES OF VARIOUS AMINO ACIDS, AND THEIR USE FOR AMINO ACID-SEQUENCE DETERMINATIONS

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The use of *iso*cyanates and *iso*thiocyanates for the determination of the sequence of amino acids in peptides was discussed in a previous paper (Evans & Reith, 1954). Aschan (1883) synthesized thiohydantoins by heating phenyl *iso*thiocyanate with glycine, alanine and leucine at 140°. The method of forming the thiohydantoic acids in alkaline solution and subsequently obtaining the thiohydantoins by acid catalysis was first used by Marckwald, Neumark & Stelzner (1891). The difficulty experienced in quantitative separation and estimation of mixtures of thiohydantoins, when only small quantities of substance are available, has led us to the synthesis of coloured thiohydantoins. Their preparation and use for the determination of the sequence of amino acids in peptides is described in

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this paper. The abbreviated nomenclature used is similar to that proposed for the *iso*cyanate derivatives (Evans & Reith, 1954). A short account has already been communicated to the Biochemical Society (Reith & Waldron, 1953).

EXPERIMENTAL AND RESULTS

Preparation of 4-dimethylamino-3:5-dinitrophenyl isothiocyanate (DDPT)

N-Acetyl-4-dimethylamino-3:5-dinitroaniline. 4-Dimethylamino-3:5-dinitrobenzoyl azide was prepared by the method already described (Evans & Reith, 1954). The azide (9·85 g.) was added to a mixture of acetic anhydride (30 ml.) and conc. H_2SO_4 (3 ml.) contained in an apparatus as described for the preparation of DDPI (Evans & Reith, 1954), and the air was displaced by CO_2. The temperature of the bath was slowly increased to 85° during 25 min., and after a further 40 min. the temperature had reached 90° when the reaction was complete. The amount of nitrogen evolved was 775·0 ml. (0°/760 mm.), theory requires 788 ml. After cooling to room temperature, acetic acid (30 ml.) was added, and the solution was then added slowly, dropwise to water (600 ml.) well stirred. The orange solid obtained was filtered off, washed with water and dried. Yield 8·72 g.; m.p. 180–184°. The substance recrystallized from toluene (200 ml.), yielded *N-acetyl-4-dimethylamino-3:5-dinitroaniline*. 6·60 g. (70%), m.p. 200–201° (decomp.) (Found: C, 45·1; H, 4·5; N, 21·1. $C_{10}H_{12}N_4O_5$ requires C, 44·8; H, 4·5; N, 20·9%.) Another 1·01 g., m.p. 170–185°, was obtained from the mother liquors.

4-Dimethylamino-3:5-dinitroaniline. The *N*-acetyl derivative (7·17 g.) was added to a solution of water (20 ml.), conc. H_2SO_4 (10 ml.) and acetic acid (20 ml.) and was heated in a boiling-water bath for 1 hr. The cooled solution was poured into water (300 ml.) when a thick orange precipitate of the amine sulphate appeared, but on addition of excess of conc. NH_4OH this was converted into a deep-red precipitate of the amine. The crude amine was filtered off, washed with water, immediately dissolved in 6N-HCl (1200 ml.) and filtered from a small amount of impurity. The acid solution was neutralized with conc. ammonia when the amine appeared as a deep-maroon precipitate (5·6 g., 93·5% yield). This was dissolved in boiling CCl_4 (300 ml.), treated with charcoal and filtered while hot. The filtrate was concentrated (to 130 ml.) and on cooling red crystals of *4-dimethylamino-3:5-dinitroaniline* were obtained, yield 5·04 g. (83%), m.p. 155–156°. Another 0·34 g. was obtained from the mother liquors. (Found: C, 42·5; H, 4·7; N, 24·7. $C_8H_{10}N_4O_4$ requires C, 42·5; H, 4·5; N, 24·8%.) The step in the purification when the amine is dissolved in 6N-HCl is particularly important in obtaining a good product though the amount of impurity removed is very small. The amine crystallized in needles having two distinct colours, deep maroon and orange-red. The former are the more stable at higher temperatures and consequently precipitated first from the CCl_4.

4-Dimethylamino-3:5-dinitrophenyl isothiocyanate (DDPT). Thiophosgene (2·64 g.; b.p. 72·5–73°) was weighed into a stoppered flask containing water (10 ml.). More water (25 ml.) was added together with $CHCl_3$ (25 ml.), and the mixture was stirred at 0° whilst 4-dimethylamino-3:5-dinitroaniline (4·18 g.) was added in $CHCl_3$ (80 ml.) during 30 min.; the mixture was stirred for a further hour at 0°, and then 1 hr. at 20°. The $CHCl_3$ layer was separated off, and the water solution extracted with two small amounts (10 ml.) of $CHCl_3$. The combined $CHCl_3$ solutions were dried overnight with $CaCl_2$ when a few yellow needles of the amine hydrochloride separated. After filtering, the $CHCl_3$ solution was evaporated *in vacuo* and the residue dried, yield 4·94 g. (99·5%), m.p. 100–103°. The *4-dimethylamino-3:5-dinitrophenyl isothiocyanate* (DDPT) was recrystallized from light petroleum, b.p. 60–80°, yield 4·58 g. (92·5%), m.p. 103–104°. (Found: C, 40·7; H, 2·9; N, 20·8; S, 11·8. $C_9H_6N_4O_4S$ requires C, 40·3; H, 3·0; N, 20·9; S, 11·9%.) Additional material was obtained from the mother liquors bringing the total yield to 97·5%. The orange-red flat needles were soluble in ether, acetone, benzene, $CHCl_3$, CCl_4, ethyl acetate, dioxan and hot light petroleum.

Reaction of DDPT with aniline. To characterize the DDPT, redistilled aniline (31 mg.) in ether (3 ml.) was treated with DDPT (89 mg.) when a red solid precipitated. The mixture was evaporated *in vacuo*, and the residue (117 mg.; m.p. 176–178°) was recrystallized from ethanol when red crystals of *N-(4-dimethylamino-3:5-dinitrophenyl)-N′-phenylthiourea* m.p. 180–181° were obtained. (Found: C, 50·1; H, 4·0; N, 19·6; S, 9·0. $C_{15}H_{15}N_5O_4S$ requires C, 49·9; H, 4·2; N, 19·4; S, 8·9%.)

Reaction of DDPT with amino acids and peptides

The reaction was carried out either in a $NaHCO_3$:water:acetone solution at a pH reading of 8·5 (glass electrode), or better in a sodium acetate:water:acetone solution at a pH reading of 8·0, which was increased at the end of the reaction to pH 9·0 by addition of alkali; in these solutions containing a large amount of acetone the recorded pH is only nominal. Sodium acetate (136 mg.) in water (6 ml.) gave a pH value of 7·3 (probably due to CO_2 in the water used); on adding acetone (12 ml.) the pH reading became 8·9 but the solution is actually more or less neutral.

Glycine (37·5 mg.; 0·5 mmole) in water (6 ml.) containing sodium acetate (136 mg.) was stirred with acetone (12 ml.), and DDPT (147 mg.; 0·55 mmole) in acetone (6 ml.) was added all at once. The pH was constantly controlled and 0·5N-NaOH was added to keep the value at 8·0 for 40 min. when the pH was raised to 9·0 for 30 min. The theoretically required amount of 1·00 ml. of alkali was needed. Evidently, when in the secondary reaction the DDPT reacts with water forming the symmetrical thiourea, the carbon oxysulphide produced is not acidic enough to titrate with alkali at the pH used. After a total period of 70 min. reaction time the solution was concentrated *in vacuo* to remove acetone, diluted with water (15 ml.) and the precipitated excess of DDPT and the symmetrical thiourea (m.p. 195–196°) were three times extracted with ether (10 ml.). The ether dissolved in the water was removed *in vacuo*, and the DDPT-glycine precipitated by a very slow addition of 0·5N-HCl (3 ml.); yield, 158 mg. (92%). A further amount of substance was extracted from the mother liquors, increasing the total yield to 99%. Recrystallization from acetone by slow addition of light petroleum, b.p. 40–60°, gave orange crystals of *4-dimethylamino-3:5-dinitrophenylthiocarbamylglycine* (DDP-thioureidoglycine), m.p. 158–159° (decomp.).

The *NN′-bis-(4-dimethylamino-3:5-dinitrophenyl)thiourea* formed as by-product by the action of water on DDPT was isolated and identified; m.p. 194–195°. (Found: C, 41·2; H, 3·7; N, 21·8; S, 6·2. $C_{17}H_{18}N_8O_8S$ requires C, 41·4; H, 3·7; N, 21·6; S, 6·5%.)

Table 1. *DDP-thioureidoamino acids and DDP-thioureidopeptides prepared by the method given in the text*

Derived from	Yield (%)	M.p. (°)*	Analysis: Found (%) C	H	N	S	Requires (%) C	H	N	S
Glycine	99	158–159	38·4	3·5	20·6	9·5	38·5	3·8	20·4	9·3
DL-Alanine	100	148–149	40·4	4·5	19·5	8·9	40·2	4·2	19·6	9·0
L-Phenylalanine	94	90–110	50·0	4·7	16·0	7·5	49·9	4·4	16·2	7·4
L-Lysine (di-DDP)	99	95–115	42·1	4·7	20·1	9·1	42·2	4·4	20·5	9·4
Glycylglycine	99	185–186	39·0	4·0	21·1	8·4	39·0	4·0	21·0	8·9
DL-Alanylglycylglycine	96	132–135	40·9	4·7	20·7	6·8	40·8	4·5	20·8	6·8

* All melting points are corrected.

Table 2. *Amino acid-DDP-thiohydantoins prepared by the method given in the text*

Derived from	Yield (%)	M.p. (°)*	Analysis: Found (%) C	H	N	S	Requires (%) C	H	N	S
Glycine	91	253–254	40·4	3·2	21·4	9·7	40·6	3·4	21·5	9·8
DL-Alanine	87	222–223	42·6	3·9	20·5	8·7	42·5	3·9	20·6	9·4
L-Phenylalanine	98	219–220	51·8	4·1	16·6	7·7	52·1	4·1	16·9	7·7
L-Lysine	77	219–220	43·6	4·7	21·2	9·3	43·3	4·2	21·1	9·6

* All melting points are corrected.

The data of the derivatives which we have prepared are given in Table 1.

In the preparation of di-DDP-thioureidolysine, the precipitation with 0·5 N-HCl gave a deep-red precipitate which was almost colloidal. This was three times extracted with ethyl acetate, the ethyl acetate solution was washed with water and evaporated *in vacuo* (<20°). The solid was dried *in vacuo* over H_2SO_4.

Preparation of DDP-thiohydantoins

The thiohydantoins were prepared from the DDP-thioureido-amino acids by treatment with either glacial acetic acid or with acetic acid:water (20:80, v/v) at about 40° for 5 days. As an example the preparation of 3-(4′-dimethylamino-3′:5′-dinitrophenyl)-2-thiohydantoin (glycine-DDP-thiohydantoin) is described. DDP-thioureidoglycine (50 mg.) was heated for 5 days in glacial acetic acid (3 ml.) at 40° and evaporated *in vacuo*. The residue was dissolved in acetone (1 ml.) and benzene (7 ml.) was added. This solution was placed on a 20 g. alumina column (10 × 220 mm.), and the chromatogram was developed with benzene:methanol (99·75:0·25, v/v; 75 ml.). The main zone was eluted with acetone and the eluent evaporated *in vacuo*; yield, 43·3 mg. (91%) of an orange crystalline substance, m.p. 253–254° (decomp.). The DDP-thiohydantoins prepared are listed in Table 2.

In the case of the phenylalanine-DDP-thiohydantoin the substance eluted from the column and crystallized contained benzene (probably 0·5 mole solvent of crystallization). The benzene was removed by dissolving the solid in acetone and evaporating the solution to dryness.

The hydantoins are deep-orange crystalline substances; the optically active amino acids were extensively racemized.

Regeneration of amino acids from their DDP-thiohydantoins

Wheeler & Hoffmann (1911) found that when hydantoins were refluxed for 5 hr. in barium hydroxide solution some 75% of the amino acids were obtained. Edman (1950) used this method in the case of the phenylthiohydantoin derivatives, and identified the amino acids by paper chromatography. We found that in the case of the DDP-thiohydantoins acid hydrolysis was preferable to the alkaline treatment. It seems that in alkaline solution the sulphur is quickly removed and the barium sulphide thus produced reacts with the nitro groups giving amino compounds. From a number of acids investigated a mixture of formic acid, acetic anhydride and perchloric acid which was used by Hanes, Hird & Isherwood (1952) for the hydrolysis of peptide bonds has proved to be very convenient. The reaction was carried out in the following way: alanine-DDP-thiohydantoin (5 mg.) was heated with 1 ml. of a mixture of formic acid (90%, w/v; 10 vol.), acetic anhydride (5·5 vol.) and perchloric acid (60%, w/v; 1·5 vol.) for 1 day at 120°. The solution was brought to pH 3 (indicator paper) by the addition of N-KOH, and was then evaporated by standing in a desiccator over NaOH. The alanine was extracted with ethanol:water (50:50, v/v), the potassium perchlorate being filtered off. Some of this solution was placed on a strip of filter paper and the chromatogram was developed in the usual way with a *n*-butanol:acetic acid:water mixture.

Spectrophotometric estimations

The absorption measurements were carried out in ethanol at concentrations between 0·1 and $1·6 \times 10^{-3}$ M for measurements at the 410 mμ. maximum, and between 0·1 and $1·6 \times 10^{-4}$ M for measurements at the other maxima (1 cm.

cuvettes, Unicam S.P. 500 Spectrophotometer). There were slight deviations from Beer's Law at the 208 mμ. maximum, but no deviations at the other maxima. The data found were as follows:

Glycine-DDP-thiohydantoin:

$\epsilon_{208\ m\mu.} = 22\,100$; $\epsilon_{262{\cdot}5\ m\mu.} = 25\,300$; $\epsilon_{410\ m\mu.} = 2260$.

Alanine-DDP-thiohydantoin:

$\epsilon_{208\ m\mu.} = 22\,400$; $\epsilon_{264\ m\mu.} = 26\,300$; $\epsilon_{410\ m\mu.} = 2240$.

Phenylalanine-DDP-thiohydantoin:

$\epsilon_{208\ m\mu.} = 25\,200$; $\epsilon_{265{\cdot}5\ m\mu.} = 25\,700$; $\epsilon_{410\ m\mu.} = 2320$.

A very interesting feature is the complete absence of a maximum at or near 360 mμ. which is characteristic of nitro compounds. The DDP-thiohydantoins actually show a minimum absorption in this region.

Chromatographic separation of the DDP-thiohydantoins

The alumina was prepared by the method already described (Flowers & Reith, 1953). As an example, the separation of a mixture of glycine-DDP-thiohydantoin with alanine-DDP-thiohydantoin proceeds as follows. Glycine-DDP-thiohydantoin (2·3 mg.) and alanine-DDP-thiohydantoin (2·2 mg.) were dissolved in acetone (0·2 ml.) and benzene (1·0 ml.) was added. This solution was adsorbed on a 20 g. alumina column (10 × 350 mm.) made with a slurry with benzene. The column was developed with benzene: methanol (99·9:0·1, v/v, 45 ml.), until the appearance of the chromatogram was 0–39 mm. white, 39–64 mm. orange, 64–78 mm. white, 78–95 mm. orange. The coloured zones were scraped out and eluted with acetone. The acetone solutions were evaporated *in vacuo*, the residues dissolved in ethanol and estimated spectrophotometrically at the 410 mμ. maximum; yield of the upper zone (glycine-DDP-thiohydantoin) 2·28 mg. (99·6% recovery); yield of the lower zone (alanine-DDP-thiohydantoin) 2·19 mg. (99·5% recovery).

The removal of N-terminal amino acids as DDP-thiohydantoins. DDP-thioureidoglycylglycine (50 mg.) was shaken at 42·5° for varying periods of time with acetic acid: water (20:80, v/v; 3 ml.). The mixture was then evaporated *in vacuo*, the residue was shaken with acetone (2 ml.) for 1 hr. after which benzene (10 ml.) and water (10 ml.) were added and the solute partitioned between these solvents. The water layer was kept for investigation. The combined benzene extracts were evaporated *in vacuo*, the residue dissolved in acetone (1 ml.), diluted with benzene (7 ml.) and adsorbed on a 20 g. alumina column. The column was developed with benzene:methanol (99·75:0·25, v/v; 100 ml.). The appearance of the column in the case of the 5-day experiment was 0–1 mm. deep red, 1–4 pale brown, 4–17 very pale yellow, 17–21 yellow, 21–35 pale yellow, 35–39 pale pink, 39–110 white, 110–240 yellow increasing to orange. The top zones were scraped out. The main zone (110–240 mm.) was eluted with acetone, the acetone evaporated *in vacuo* and the residue consisting of glycine-DDP-thiohydantoin was estimated gravimetrically; the yields were as follows. 2 days 19·9 mg. (49%), 2·75 days 26·2 mg. (64·5%), 3 days 29·8 mg. (73%), 4 days 35·3 mg. (87%), 5 days 37·6 mg. (92·5%). The water layer (see above) which contained glycine was titrated with alkali by the formol titration method (glass electrode). For the 2·75-day reaction the yield of glycine was 6·3 mg. (67%), for the 5-day reaction 9·1 mg. (97%).

An experiment with DDP-thioureidoalanylglycylglycine (50 mg.) carried out in a similar way gave the following yields of alanine-DDP-thiohydantoin: in the experiment lasting 1 day 30·6 mg. (85%), 2 days 30·6 mg. (85%), 4 days 31·0 mg. (86%). The water layer containing the glycylglycine was in each case titrated with alkali by the formol method. For the 1-day reaction the yield of glycylglycine was 11·7 mg., 2 days 11·5 mg., 4 days 12·4 mg. (the mean is 85%), which corresponds quite well with the quantity expected from the 85% yield of alanine-DDP-thiohydantoin.

Sequence determination in alanylglycylglycine

Alanylglycylglycine (20·3 mg.) was treated with DDPT (40 mg.) as described above. After acidifying the water solution with 0·5N-HCl, the DDP-thioureidoalanylglycylglycine was extracted with ethyl acetate (5 ml.) five times. The combined ethyl acetate extracts were evaporated *in vacuo*, and the residue was shaken with water:acetic acid (80:20, v/v; 3 ml.) for 1 day at 42·5° in a 'Quickfit' flask (50 ml.) closed with a cone carrying a tap. The cone was strongly held in position by steel springs, and before shaking, the flask was evacuated to 100 mm. Hg. The solution was evaporated *in vacuo*, acetone (2 ml.) was added and the mixture was shaken for 1 hr.; benzene (10 ml.) and water (10 ml.) were then added and the solute partitioned between these two solvents. The benzene solution was evaporated *in vacuo*, the residue was dissolved in acetone (1 ml.), diluted with benzene (7 ml.) and adsorbed on a 20 g. alumina column. The column was developed with benzene: methanol (99·9:0·1, v/v); after separation the upper bands were scraped out, and the main zone eluted with acetone. The acetone solution yielded 28·0 mg. (82·5%) of alanine-DDP-thiohydantoin which was identified by its melting point and by paper chromatography.

The water solution, which contained glycylglycine, was then treated in an analogous manner. The yield of the first glycine-DDP-thiohydantoin was 23·3 mg. (71·5% of yield calc. for alanylglycylglycine), and that of the second was 19·7 mg. (60·5%).

DISCUSSION

Although 4-dimethylamino-3:5-dinitrophenyl *iso*cyanate is an excellent reagent for the determination of *N*-terminal amino acids (see preceding paper), it was not entirely satisfactory for the determination of the sequence of amino acids by successive applications to a peptide. The use of DDPT described in this paper overcomes this difficulty. It gives excellent yields of DDP-thioureidoamino-acids and peptides under mild conditions, and these are converted into DDP-thiohydantoins under much milder conditions than were necessary for the DDP-ureidoamino acids and DDP-ureidopeptides.

The DDP-thiohydantoins are deeply coloured compounds which can be separated from one another by chromatography and can easily be estimated by colorimetric techniques. By subsequent acid hydrolysis of these thiohydantoins the parent free amino acid can be regenerated and its identity can be checked by paper chromatography.

DDPT is thus a suitable reagent for the determination of the amino-acid sequence in peptides by a process of step-wise degradation from the *N*-terminal end of the chain, and has in fact been successfully applied for this purpose. The conditions under which the *N*-terminal amino acid splits off as a substituted DDP-thiohydantoin are so mild that other peptide bonds are not split. The yields of the thiohydantoins vary between 80 and 90 % according to the experimental conditions and the type of the thiohydantoin. We found that the yield of the residual peptide (subject to the next step in the sequence determination) is practically equivalent to the yield of the thiohydantoin. Consequently a small but calculable fraction of the DDP-thioureidopeptide is lost during the process of the elimination of the substituted *N*-terminal group.

SUMMARY

1. A deep-orange *iso*thiocyanate, 4-dimethylamino-3:5-dinitrophenyl*iso*thiocyanate (briefly called DDPT) was synthesized.

2. It was found that this *iso*thiocyanate reacts with amino acids or peptides dissolved in water:acetone (1:3, v/v) at the apparent pH 8–9, giving orange thiocarbamyl derivatives (briefly called DDP-thioureidoamino acids or DDP-thioureidopeptides, e.g. DDP-thioureidoglycine) in practically quantitative yields.

3. By the treatment of the DDP-thioureidoamino acids with glacial acetic acid (in the case of DDP-thioureidoglycine) or with water:acetic acid (in the case of other DDP-thioureidoamino acids) the corresponding substituted 2-thiohydantoins (briefly called amino acid-DDP-thiohydantoins, e.g. glycine-DDP-thiohydantoin) are formed in good yields (80–90 %).

4. By treatment of DDP-thioureidopeptides with water:acetic acid, the *N*-terminal amino acid residue splits off as an amino acid-DDP-thiohydantoin which can be isolated in a pure state by adsorption chromatography; the residual peptide can be treated with DDPT and the process repeated to determine the sequence of amino acids in the peptide.

5. As an example the sequence determination on alanylglycylglycine is described.

6. The amino acid-DDP-thiohydantoins can be hydrolysed to the parent amino acids and these can be identified by the use of paper chromatography.

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The Extent of Conversion of Food Protein to Microbial Protein in the Rumen of the Sheep

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Interest in the role of ruminal micro-organisms in the protein metabolism of ruminants stems from the hypothesis of Zuntz (1891) that these organisms might utilize dietary non-protein nitrogen (N.P.N.) for their growth, with resultant synthesis of protein which would become available to the host. Numerous experiments have established the validity of this view (for a review of the extensive literature in this field see McNaught & Smith, 1947); indeed, Loosli, Williams, Thomas, Ferris & Maynard (1949) were able to maintain growth in sheep on a diet in which virtually all the nitrogen was supplied by urea.

In spite of the activity in this field, comparatively little attention has been given to the possibility of microbial digestion of food protein in the rumen. In normal ruminant diets most of the nitrogen occurs in the form of protein. The facts that secretory glands do not occur in the rumen, that the saliva contains no proteolytic enzyme (Wegner, Booth, Bohstedt & Hart, 1940) and that the rumen contents are strongly proteolytic (Sym, 1938) suggest that ruminal organisms play an active part in the digestion of protein. Pearson & Smith (1943) observed breakdown of protein by rumen liquor *in*

vitro, and McDonald (1948*a*, 1952) showed that soluble protein was extensively degraded in the rumen with the formation of ammonia.

The extent to which ruminal microbes convert fodder protein into the proteins of their own structures has been the subject of several speculations, though little experimental effort has been directed to the problem.

Schwarz (1925) concluded that the 'greatest part' of the protein requirement of cattle was met by the digestion of microbial protein derived from fodder protein. However, his data, confined to analyses of rumen contents, do not warrant such a conclusion, since he found on the average 60% of the total nitrogen of rumen contents in the fodder residues and 32% in the micro-organisms. His views were opposed by Mangold & Schmitt-Krahmer (1927), but the evidence they adduced was equally unconvincing. Mitchell & Hamilton (1929) criticized these speculations and pointed out that it was not enough to perform analyses on the rumen contents of slaughtered animals, but that it was necessary to estimate the daily production of microbial protein in the rumen.

Ferber & Winogradowa-Federowa (1929) calculated that rumen protozoa provided 2% of the protein requirements of the sheep; this estimate was based on their observation that at any given time an average of 7% of the infusoria of rumen contents were in process of dividing, and they assumed that this was also the daily reproductive rate. Hungate (1942) considered this value too low; he found that, in culture, the protozoan *Eudiplodinium neglectum* had a division rate of about once per day and hence calculated that the protozoa provided about 20% of the host's daily protein requirements.

Köhler (1940) attempted to assess the daily output of microbial nitrogen from the rumen of cattle by comparisons of bacterial numbers or weight in the contents of the rumen and the duodenum, but found his methods inadequate to yield acceptable results.

Thaysen (1945) estimated that a minimum of 180 g./day of microbial protein passed from the rumen of cattle. He based his calculations on analyses of centrifuged rumen liquor and on the assumption that 100 l. of rumen liquor flowed from the rumen to the abomasum each day. McNaught & Smith (1947) used the data from the *in vitro* experiments of Pearson & Smith (1943) to estimate that 100–150 g. of bacterial protein were formed/day in the rumen of cattle; alternatively, using the data of Schwarz (1925) and an assumed rate of flow of digesta from the rumen of the ox of 40 kg./day, they calculated that 75 g. of bacterial protein would be formed/day.

Moir & Williams (1950) observed, in sheep, a constant increase in the numbers of ruminal micro-organisms with increasing protein intakes, suggesting that a constant proportion of the food protein was converted into bacterial protein; they calculated that approximately 50% of the dietary protein was converted into bacterial protein, but subsequently recognized (Moir, 1951) that such a calculation could not be made from their data.

Indirect evidence for the contribution of micro-organisms to the protein metabolism of the host animals has accrued from estimates of the biological value of protein for ruminants. Johnson, Hamilton, Mitchell & Robinson (1942) and Miller & Morrison (1942) noted a relative constancy in the biological values of the proteins they examined. It was suggested (Johnson, Hamilton, Robinson & Garey, 1944) that, according to the capacity of the rumen microbes to utilize nitrogen, all feeds would have the biological value of microbial protein (namely about 60%) whilst any protein not utilized by the micro-organisms would have a biological value approximately the same as that found for non-ruminants. The later observations on biological value made by Lofgreen, Loosli & Maynard (1947) and by Hamilton, Robinson & Johnson (1948) suggest that the proportions of dietary and microbial protein which actually become available to the host might vary considerably on different diets.

A survey of the literature thus reveals that it is likely that the growth of micro-organisms (bacteria and protozoa) in the rumen results in a significant degree of conversion of the animal's food nitrogen into microbial protein, but that the extent of this conversion and the factors influencing it are not evident. The present work comprises an attempt to develop procedures for direct experimental approach to the problem. A preliminary account of this work has previously been published (McDonald, 1948*b*).

EXPERIMENTAL

Methods

The determination of the quantity of microbial protein which passes from the reticulum into the abomasum, and thence through the remainder of the intestinal tract presents two major requirements: the collection of digesta received by the abomasum, and the analysis of the digesta for microbial proteins and/or the undigested food protein.

The collection of the digesta presents numerous difficulties. No means have yet been devised for sampling digesta at the entrance to the omasum or as it leaves this viscus to reach the abomasum. No difficulty is entailed in collecting the digesta from the abomasum via a permanent fistula, but this approach has not been favoured as it was found necessary to collect the whole of the contents of the organ in order to obtain satisfactory sampling, and it seemed probable that the artificial emptying of the abomasum would influence the rate of flow of digesta from the rumen and reticulum, and the rate of secretion of gastric juice. On these grounds it appeared desirable to collect the abomasal digesta immediately after it passed through the pylorus.

Table 1. *Recovery of zein added to abomasal contents*

(For this test abomasal contents were taken from a sheep receiving a diet in which casein replaced the zein of the experimental diet. The excess recovery of zein is due to the presence of traces of nitrogen, probably lipid N, soluble in 80% ethanol but not in 5% trichloroacetic acid.)

Abomasal contents (ml.)	Zein N added (mg.)	Total N found (mg.)	Total N after removal of zein (mg.)	Zein N by difference (mg.)	Zein N recovered (%)
20	0	7·2	—	—	—
20	5·25	12·3	6·8	5·5	105
20	10·5	17·7	6·8	10·9	104
20	15·75	23·0	6·8	16·2	103
20	21·0	28·2	6·9	21·3	101

Theoretically, any difference between the proteins of the food and of the ruminal microbes could be used as the basis of an analytical method for the determination of the proportions of microbial protein and food protein in the digesta. In practice, the task presents many problems, the most important of which are the mixed nature of the microbial proteins and the difficulty in providing suitable diets in which a single protein is the sole source of nitrogen. In these preliminary experiments, therefore, a diet based on the protein zein was used and the analyses were based on the solubility of zein and the insolubility of microbial proteins in aqueous ethanol. In a single experiment, the fact that zein contains no lysine was used as the basis of analytical determination of food protein in the digesta.

Despite the obvious criticism that these experimental techniques could not be expected to reflect accurately the events occurring in the digestion of natural diets, it was felt that preliminary experiments would be worth while as a basis for more extended studies.

The experimental sheep were provided with fistulae into the dorsal sac of the rumen and into the duodenum immediately distal to the pylorus; the fistulae were closed with suitable cannulae. At appropriate times, samples of the rumen liquor and of the abomasal digesta were collected for analysis.

In the first experiment reported, the daily ration comprised: zein 110 g., rice starch 250 g., glucose 80 g., cellulose 230 g., molasses 40 g. and straw chaff 150 g. In this diet, zein supplied 94% of the total N. The diet was supplemented with NaCl, calcium phosphate, and vitamins A and D. The feeds were prepared as a moist mash so that the powdered constituents were held in intimate contact with the fibrous cellulose and straw; the food was given once daily and was usually completely consumed within 2 hr. of feeding.

In the second experiment, the diet consisted of: straw chaff 500 g., starch 100 g. and zein 75 g.; the zein supplied 82% of the total protein in the diet.

Methods of analysis

Total nitrogen was determined by the Kjeldahl method using the procedure of Chibnall, Rees & Williams (1943) or that of Hiller, Plazin & Van Slyke (1948). Protein other than zein was precipitated by dilution of the sample with ethanol to give a concentration of approximately 80% (v/v). After filtration, zein was separated from the ethanolic filtrate by boiling off the ethanol under reduced pressure; the residue was extracted with 5% (w/v) trichloroacetic acid (TCA) and filtered; the nitrogen in the filtrate was designated non-protein nitrogen (N.P.N.) and the zein N was calculated as the difference between total N of the ethanolic filtrate and N.P.N. In Table 1 are given the results of a test for the recovery of zein added to a sample of abomasal contents.

Lysine was estimated by the decarboxylase method of Gale (1945).

RESULTS

Experiment 1

The results of eight series of analyses of the abomasal contents are given in Table 2, and the average values for all samples taken at a given time after feeding are plotted in Fig. 1. The data were analysed by the *t* test for the significance of difference of means. The analysis revealed no significant differences between the mean values during the first 4 hr. after feeding; the values at 7 and 14 hr. are significantly greater than the pre-feeding levels ($P = 0{\cdot}01$). It is therefore probable that the proportion of zein slowly increases to reach a maximum many hours after feeding and slowly returns to the original value after 24 hr.

It is not possible to use these data for an accurate estimate of the total fraction of zein which leaves the rumen undigested. With certain assumptions, however, an approximate estimate may be made; these assumptions are:

(1) That in the abomasum, none of the zein is digested to fragments sufficiently small to be soluble in dilute TCA. This assumption probably introduces very little error. It has been shown by Laine (1944) that pepsin breaks down zein very slowly. The low values for N.P.N. in the rumen liquor (mentioned below) indicate that the micro-organisms are capable of taking up the products of digestion of the food protein more rapidly than their proteolytic enzymes can degrade the zein; it is probable, therefore, that the digesta contain no appreciable quantity of TCA-soluble fractions of zein derived from microbial digestion in the rumen.

(2) That all the dietary protein leaves the rumen either as microbial protein or as undigested food protein. It has been previously shown (McDonald, 1952) that zein is very much more slowly attacked in the rumen than proteins like casein, gelatin or grass protein, with the result that the concentration of ammonia in the rumen is low when sheep are fed

Table 2. *Results of analyses of abomasal contents for total protein* N *and zein* N

Series no.	1	2	3	4	5	6	7	8	
Days on exptl. diet ...	10	16	20	26	36	10	29	6	
	Zein N as % protein N in abomasal contents								
Hr. after feeding									Mean
24	38	26	34	37	48	65	60	44	44
1	43	26	30	50	42	38	42	49	40
2	43	36	35	42	46	56	63	54	47
4	59	51	57	56	34	45	58	46	51
7	63	62	66	44	68	—	47	62	59
14	—	—	—	50	62	76	76	64	66
24	42	35	46	44	49	57	55	47	47
						Mean of all values			49·4

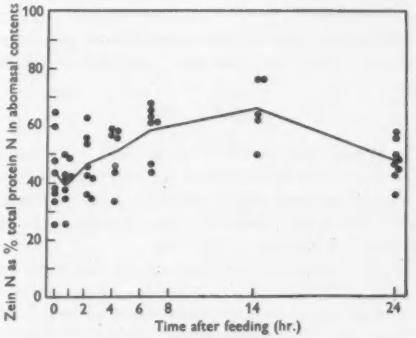

Fig. 1. Graph showing changes in content of zein in abomasal contents.

a zein diet. For convenience of calculation, therefore, it has been assumed that loss of nitrogen from the rumen (as absorbed ammonia) is balanced by the addition of nitrogen to the rumen by saliva.

(3) That the rate of flow of digesta from the abomasum to the duodenum is constant. Phillipson (1952) has discussed the variations which occur in this rate of flow; although complete cessation of flow for periods up to 30 min. were observed, substantial flow of digesta usually occurred whenever collections were made; the rate of flow was not definitely correlated with time of day or with the act of feeding. Since the diurnal variation in the mean percentage of zein in the total protein of the duodenal contents was not great, it seems likely that the assumption of a constant rate of flow would not introduce a large error.

(4) That the protein other than zein consists solely of microbial protein. Evidence has not yet been obtained to enable an estimate to be made of the magnitude of the error introduced by this assumption. There will also be present protein derived from (*a*) the undigested fragments of dietary straw, which however contributes only 5% of the total protein in the diet; (*b*) from the gastric juice; the error from this source is probably not great, as analysis of gastric juice, obtained from an abomasal fistula after emptying the entire stomach, showed a protein content of only 4 mg. N/100 ml.; (*c*) a small amount of protein may also be derived from undigested protein of the saliva and of desquamated epithelial cells from the mucosa of the stomach. The protein from these various sources will constitute an error increasing the value for microbial protein; this error will, however, be compensated by the fact that some of the microbial protein (and especially that of the delicate protozoa) will be degraded by peptic digestion in the abomasum to fragments soluble in 80% ethanol and hence estimated as N.P.N.

On the basis of these assumptions the following calculation can be made as a first approximation: the area of the graph in Fig. 1 below the mean curve indicates that 56% of total protein nitrogen in the abomasal digesta is comprised of zein; and since zein provided 94% of the dietary protein, it is evident that approximately 40% of the zein had been converted, in the rumen, into microbial protein.

Experiment 2

In this experiment advantage was taken of the fact that zein contains no lysine; hence if the dietary zein were used by the ruminal micro-organisms for growth, they would have to synthesize their own lysine. The difference in lysine contents of the diet and the abomasal contents could therefore be exploited to estimate the output of microbial protein from the rumen. Abomasal contents were collected for periods of 1 hr. and samples of rumen liquor were taken at the same time.

The intake of protein nitrogen was 13·4 g./day, comprising 11·1 g./day of zein nitrogen with 2·3 g. of protein nitrogen in the straw. The lysine present in the straw represented 0·16 g. nitrogen/day so that the percentage of lysine nitrogen in the total dietary protein nitrogen was 1·2%. The proteins of the rumen liquor were taken as a satisfactory approximation to 'microbial proteins' since the preparation of rumen liquor by straining through muslin removed all but traces of straw fragments

and the adherent zein; the absence of zein was demonstrated by the correspondence between the N.P.N. values obtained after precipitation with ethanol and aqueous acid respectively. The microbial proteins of the rumen liquor were found to have a lysine nitrogen content of approximately 7% of the protein nitrogen; this value may be compared with values, calculated to the same basis, of 6·8% (Loosli *et al.* 1949) and 6·1% (Duncan, Agrawala, Huffman & Luecke, 1953) for microbial proteins of ruminants fed on diets in which urea was the sole source of nitrogen. The protein of the mixed abomasal contents, comprising undigested straw-protein, zein and microbial protein exhibited lysine nitrogen contents of 3·9–5·7%. Whilst accurate calculations cannot be made from these data, it is evident, following the same assumptions as previously given, that some 40–50% of the ingested zein must have been converted in the rumen into microbial protein. This result is in accord with the results obtained in the previously recorded experiment.

DISCUSSION

The need for information on the capacity of the ruminal micro-organisms to utilize dietary nitrogen for their growth has been recognized by numerous workers, for this represents an important facet of the nitrogen metabolism of the ruminant. It is clear that the nitrogenous substances absorbed by the host are not merely those of the diet, as in the monogastric animal, but a mixture of dietary constituents, products of microbial metabolism in the rumen and the constituents of the micro-organisms themselves. In this connexion, interest centres chiefly on the proteins which ultimately become available for absorption by the ruminant.

No definite evidence has previously been reported which would indicate the extent to which dietary protein is converted into microbial protein in the rumen and hence the proportions of microbial and dietary proteins which leave the rumen. The present work indicates that a direct experimental approach to the problem is practicable. For this preliminary work, zein was chosen for the analytical advantages it provided; it is desirable, however, that the work should be extended in an effort to study the fate of proteins in ordinary diets and at various levels of feeding. The difficulties inherent in the task are numerous, and it will be clear from the assumptions necessarily made in this paper that much collateral work on the physiology of digestion in the ruminant will be required before a full understanding of the role of micro-organisms in the digestion of protein in the ruminant is attained.

Zein is not a very satisfactory protein for use as the major fraction of the dietary protein; it is highly insoluble in aqueous solutions and when a suspension is warmed to body temperature (39° for the rumen) it forms a glutinous, fibrous mass, thus reducing the surface area available for enzymic attack. In addition the protein lacks lysine and tryptophan, and hence these amino acids must be synthesized by the micro-organisms. The effect of these properties is reflected in the finding (McDonald, 1952) that zein is slowly attacked in the rumen and that, in contradistinction to ordinary diets or those containing casein and gelatin, ammonia does not accumulate in significant amounts in the rumen. For these reasons, it seems likely that the observed degree of conversion of zein into microbial protein is probably less than would occur in animals fed on ordinary diets.

The application of these experimental procedures to other dietary proteins is at present being undertaken.

SUMMARY

1. When sheep were fed a partially purified diet to which zein contributed 94% of the total nitrogen, it was found that approximately 40% of the zein was utilized by ruminal micro-organisms for the synthesis of their own proteins.

2. This conclusion was based on the separation of the ethanol-soluble protein, zein, from the microbial protein in the digesta received by the duodenum. The conclusion was supported by the result of an experiment based on analyses of lysine in the foodstuffs, ruminal micro-organisms and abomasal contents.

3. Reasons are advanced for suggesting that, in normal dietary regimes, a higher proportion of food protein would be converted in the rumen into microbial protein.

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Purification and Properties of Phosphoprotein Phosphatase from Ox Spleen

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Harris (1946) detected for the first time, in frog's eggs, an enzyme capable of effecting a specific dephosphorylation of phosphoproteins and accordingly termed it phosphoprotein phosphatase. A similar enzyme was found to be present in rat tissues by Feinstein & Volk (1949) who also studied some of its properties. The enzyme was not activated by metal ions, nor did it lose its activity when dialysed. A characteristic property of the enzyme was its activation by reducing agents. This was, however, contradicted by Norberg (1950) who, in his experiments, could find no activation. On account of the divergent views on this question, and since the previous investigators used crude tissue extracts as the enzymic material in their studies, the problem has been re-investigated with a purer enzyme preparation. The object of the present study was also to establish the existence of phosphoprotein phosphatase in animal tissues as an independent enzyme quite distinct from the phosphomonoesterases. Accordingly a method has been evolved for the purification of the enzyme from ox spleen. The final product obtained represents a 200-fold purification and can be considered to be the purest specimen of the enzyme so far obtained.

EXPERIMENTAL

Materials

Substrates. Casein prepared according to the method of Cohn & Hendry (1930) was used routinely as substrate. Phosvitin and vitellin were prepared from egg yolk according to the methods of Mecham & Olcott (1949) and of Calvery & White (1931), respectively. Phosphopeptone was prepared in the form of its barium salt from a peptic-tryptic digest of casein according to the method of Damodaran & Ramachandran (1941). The glycerophosphate employed was the β-isomer obtained from British Drug Houses Ltd. (B.D.H.).

Activator. Thioglycollic acid (B.D.H.) was used as an activator, unless otherwise stated. The enzyme was found to exert its maximum activity in the presence of 0·001 M-thioglycollic acid.

Buffer. Michaelis veronal-acetate buffer.

Methods

Measurement of enzyme activity. The test mixture employed for the measurement of the enzyme activity was made up as follows: 1 ml. of the mixture contained 10 μmoles casein P, 1 μmole activator, 20 μmoles buffer at pH 6 and varying amounts of the enzyme solution. The mixtures were incubated at 37° for 30 min. After deproteinization with trichloroacetic acid (TCA), the activity was followed by the estimation of the liberated inorganic P by the method of Fiske & SubbaRow (1925). Controls with casein and water and blank values with water and sample were run at the same time.

Unit of enzymic activity. This was defined as the amount of enzyme catalysing the splitting of 1 μg. of inorganic P/min. at 37° and at pH 6 from a test mixture containing 10 μmoles of casein P/ml.

Specific activity. This was expressed in units/mg. protein N.

Determination of protein nitrogen. The protein was first precipitated from the test solution by TCA. When washed free from ammonia the N content was determined by the micro-Kjeldahl method (Pregl, 1945).

RESULTS

Purification of phosphoprotein phosphatase

Ox spleen was preferred as starting material for the preparation of the enzyme on account of its ready availability and relative abundance.

Extraction of the enzyme. Spleens from freshly slaughtered oxen were removed and conveyed from the slaughter house

Table 1. *Purification of phosphoprotein phosphatase from ox spleen*

Step	Fraction	Volume (ml.)	Total activity (units)	Overall yield (%)	Specific activity
1	Original saline extract	350	3410	—	1·35
2 and 3	Fraction *A*	130	1023	30	3·25
2 and 3	Fraction *B*	27	1087	32	5·19
4	Fraction A_1	10	35	1·0	—
	Fraction A_2	50	520	15	—
	Fraction *C*	25	1324	39	37·3
5	Fraction *D*	25	417	12·2	28·5
	Fraction *E*	12·5	675	19·8	254

mixed with ice. After a preliminary mincing of the tissue in a meat chopper, a weighed amount of the minced tissue was ground well in a Waring Blendor with 2·5 times its weight of 0·5 M-NaCl, buffered at pH 5·8. The use of this medium facilitates the almost quantitative extraction of the enzyme besides ensuring its stability. The tissue suspension was centrifuged for 15 min. at 2000 rev./min. The supernatant was opalescent and reddish brown in colour.

Ammonium sulphate fractionation. The saline extract was cooled to 10° and solid $(NH_4)_2SO_4$ was added gradually with mechanical stirring to 0·3 saturation (21·9 g./100 ml.). Most of the protein was separated at this concentration (fraction *A*). The precipitate was separated by filtration through fluted filter papers and allowed to drain in the ice chest (10°). The filtrate was next treated with $(NH_4)_2SO_4$ to 0·6 saturation and the precipitate separated as before (fraction *B*). A portion of the filtrate from 0·6 saturation was treated with $(NH_4)_2SO_4$ to full saturation. The precipitate contained very little of the activity and hence was discarded.

Precipitation of the enzyme by dialysis. Fractions *A* and *B* were taken up separately in water and dialysed at 10° in cellophan sacks against frequent changes of distilled water. A flocculent precipitate separated in each case at the end of 24 hr. and settled down in the sacks after the completion of dialysis in about 72 hr. The precipitates were separated by centrifugation, taken up separately in 0·5 M-NaCl at pH 5·8, and the activities of the different fractions were estimated as usual. The precipitates in each case were found to contain most of the activities of the respective fractions, whereas the supernatants had negligible activities.

Refractionation with ammonium sulphate of 0·3 saturation fraction. The precipitate which separated on dialysis of fraction *A* was dissolved in a suitable amount of 0·5 M-NaCl and filtered through fluted filter paper. The filtrate was subjected to a refractionation with $(NH_4)_2SO_4$. This time most of the activity was found to have gone over to the 0·6 fraction (fraction A_2). The 0·3 fraction (fraction A_1) contained little activity and consequently was rejected. The appreciable amount of activity found in the 0·3 fraction during the first fractionation (fraction *A*) is obviously due to the adsorption of the enzyme on the bulky precipitate formed, rather than due to any precipitation of the enzyme at this salt concentration.

Fraction A_2 was taken up in water and dialysed as before. The precipitate which separated during dialysis was added to the corresponding fraction obtained in the first $(NH_4)_2SO_4$ fractionation. The pooled precipitates were extracted once with 0·5 M-NaCl. The extract (fraction *C*) containing high phosphatase activity was finally subjected to an acetone fractionation.

Table 2. *Relative action of the different fractions towards casein and glycerophosphate*

(Activities of the crude spleen extract and of the purified enzyme were tested on 0·5 and 0·1 ml. samples, respectively. Final concentration of substrates corresponded to 10 μmoles of organic P/ml.)

Expt. no.	Enzyme source	Specific activity with: Casein	Specific activity with: Glycerophosphate
1	Crude spleen extract	1·07	0·52
	Final purified product	190·5	10·04
2	Crude spleen extract	1·10	0·61
	Final purified product	205	23·33

Fractionation with acetone. To a measured amount of the saline extract (fraction *C*), cooled in a freezing mixture of ice and salt, ice-cold acetone (B.D.H., A.R. grade) was added dropwise with vigorous mechanical stirring until the acetone concentration was 50%. The precipitate formed was separated on a centrifuge. Further quantities of acetone were added to the supernatant as before till the acetone concentration reached 66%. The precipitate formed was removed by centrifugation. The two fractions were dissolved separately in suitable amounts of 0·5 M-NaCl, centrifuged and the activities of the water-clear supernatants (fractions *D* and *E*) were estimated as usual.

The results obtained in a typical fractionation experiment are presented in Table 1. The precipitate separating between 50 and 66% acetone concentration (fraction *E*) will be seen to have the highest activity, representing approximately a 200-fold purification. This fraction has been used throughout this work for the study of the properties of the enzyme and will be referred to as the purified enzyme.

The enzymic properties of phosphoprotein phosphatase

Specificity

Relative action of the crude and purified enzyme preparations towards casein and glycerophosphate. The object of the experiment was to see whether phosphoprotein phosphatase could be differentiated from acid phosphomonoesterases on the basis of its action on the different substrates. The results presented in Table 2 show that on purification of the enzyme there is a marked decrease in activity with

glycerophosphate as substrate and an increase with casein. The hydrolysis of the two substrates must hence be assumed to be brought about by two different enzymes.

Action of the purified enzyme on other phosphoproteins. Since the enzyme has a preferential action on casein, it would be of interest to see whether it can attack the other phosphoproteins with equal vigour. It is evident from the results given in Table 3 that both phosvitin and vitellin are dephosphorylated by the enzyme, though not to the same extent as casein. The figures for vitellin are perhaps too low, since only a part of the substrate was present in the dissolved state in this case. No appreciable dephosphorylation took place with phosphopeptone. It is of interest to note that neither phosphopeptone nor phosphoserine is attacked by this enzyme (Norberg, 1950; Feinstein & Volk, 1949). Evidently the enzyme cannot attack casein after considerable proteolytic degradation.

Kinetics of enzymic action

Fig. 1 presents the time/activity curves of our purified enzyme preparation. A linear relationship is found to hold good in the initial stages in both the preparations studied. The main reason for the slowing down of the reaction may be the influence of the split products on enzymic action. It is of interest to note in this connexion that the activity of the enzyme is diminished by added inorganic phosphorus (Norberg, 1950).

Effect of substrate concentration on enzymic activity. The dependence of enzyme activity upon substrate concentration is shown in Fig. 2. The activity of the enzyme is found to increase at first but remains constant at a substrate concentration corresponding to about 10 μmoles of casein phosphorus/ml. Treatment of the results according to the method of Lineweaver & Burk (1934) gives a Michaelis constant of 2 mM of casein phosphorus. This figure is somewhat lower than the value of 3 mM obtained by Norberg (1950) for rat-spleen phosphatase.

Optimum pH. The effect of pH on the rate of the enzymic hydrolysis of casein is shown in Fig. 3. The optimum pH lies in the region of pH 6·0. Hydrolysis is negligible below pH 4·5 and above pH 7.

Table 3. *Action of the purified enzyme on some phosphoproteins*

(0·1 ml. of the purified enzyme preparation was used for each experiment. All the substrates were present in a final concentration corresponding to 10 μmoles/ml. of organic P. Phosvitin and vitellin were weighed directly into the reaction tubes. Other substrates were added in the form of solution.)

Substrate	μg. of inorg. P liberated in 30 min. by: Enzyme preparation *A*	Enzyme preparation *B*
Casein	121	130
Phosvitin	57	74
Vitellin	30	36
Phosphopeptone*	12	—

* Quantitatively freed from barium.

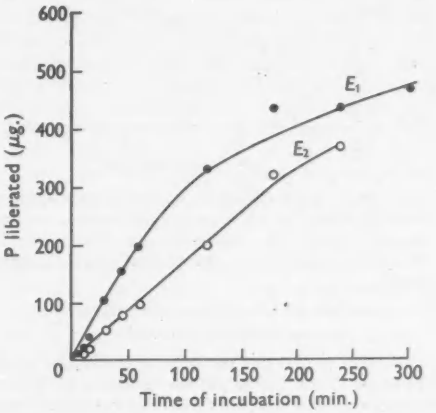

Fig. 1. Time/activity curves of phosphoprotein phosphatase. Conditions as in test mixture (see Experimental). E_1 and E_2 are curves for two different enzyme preparations having specific activities of 254 and 86 units, respectively. 0·1 ml. of the preparation was used for each experiment.

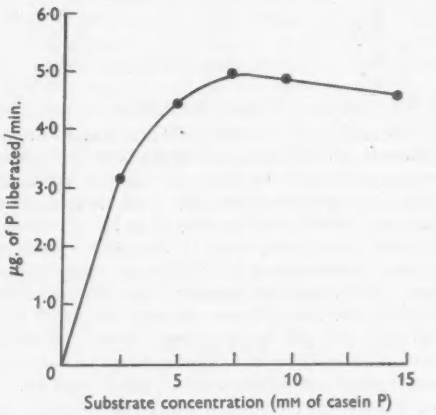

Fig. 2. Substrate concentration and enzyme activity. Conditions as in test mixture. 0·1 ml. of the purified enzyme was used for each experiment. Incubation time, 30 min. at 37°.

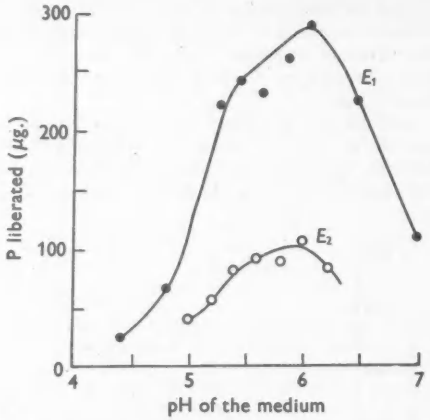

Fig. 3. Effect of pH on enzyme activity. Conditions as in 'test mixture'. 0·1 ml. of the purified enzyme was used for each experiment. Incubation time, 30 min. at 37°. E_1 and E_2 are curves for two different enzyme preparations.

Table 4. *Stability of the enzyme at various* pH *values*

(A sample (0·1 ml.) of the enzyme preparation mixed with buffer of the desired pH was incubated for 1 hr. at 37° with and without activator as indicated below. After readjusting the pH to 6·0, activities of the preparations were assayed as usual with casein as substrate.)

pH of incubation	Loss in activity (%): Thioglycollic acid added before incubation	Loss in activity (%): Thioglycollic acid added after incubation
5·0	1·0	0
6·0	18·6	0
7·0	64·3	24·3
8·0	82·9	32·9

Effect of pH *on stability of the enzyme*

The stability of the enzyme preparation at four different pH values has been studied. Two sets of experiments were carried out. In one series, the enzyme preparation, brought to the desired pH by means of buffer, was incubated in the presence of activator at 37° for 1 hr. In the second series the activator was added only after the completion of this preliminary incubation. The solutions were assayed for phosphatase activity as usual after bringing the pH back to 6·0. From the results presented in Table 4 it appears that the enzyme is more stable at pH 5 than at pH 6, and that it undergoes appreciable inactivation at pH 7 and above. Further, thioglycollic acid has no protective action when it is added before incubation. It can, however, bring about a marked reactivation when it is added after incubation.

Table 5. *Effect of metal ions on enzyme activity*

(0·1 ml. of the pure enzyme preparation was incubated with the indicated metal ion and buffer at pH 6 for 15 min. at 30°. Activity was then measured as usual with casein as substrate. No activator was added in the case of the heavy metal ions.)

Substance added	Molarity	Activation (+) or inhibition (−) (%)
NaCl	0·01	−3·0
K_2SO_4	0·01	−8·3
$MgCl_2$	0·01	−4·2
$MnSO_4$	0·01	+2·8
$CaCl_2$	0·01	−8·3
$BaCl_2$	0·01	+2·8
$CuSO_4$	0·01	−75·0
—	0·001	−65·5
—	0·00025	−69·0
Lead acetate	0·01	−65·5
$HgCl_2$	0·01	−28·0
$ZnSO_4$	0·01	−100·0

Activators and inhibitors of the enzyme

Effect of metal ions. The results summarized in Table 5 show the effect of various metal ions on the activity of the enzyme. Thioglycollic acid was used as an activator for the first six metal ions. No activator was added in the case of heavy metal ions, since if the latter act by combining with the —SH group of the enzyme protein, the presence of thiol substances will naturally counteract their action. From the results shown it will be seen that whereas none of the metal ions produce any appreciable activation of the enzyme, inhibition is observed with ions of all heavy metals. Copper and zinc are especially inhibitory, the former ion exerting its inhibitory effect even when present at a concentration of $2{\cdot}5 \times 10^{-4}$ M. Inhibition at such low concentration suggests that the sulphydryl group of the enzyme protein may be the point of attack in these cases.

Effect of oxidizing and reducing agents. Feinstein & Volk (1949) have reported a marked activation of rat-spleen phosphoprotein phosphatase by reducing agents and an inhibition by oxidizing agents. Norberg (1950), however, could find no activation of the enzyme in the presence of 0·01 M ascorbic acid. Table 6 gives a list of oxidizing and reducing agents employed in the present investigation. All these reagents were present in a final concentration of 0·001 M and were not found to interfere in the colorimetric estimation of phosphorus. This was confirmed by carrying out the estimation in the absence of these reagents according to the method of Delory (1938).

From the results presented in Table 6, it will be seen that while cyanide produced an inhibition, all reducing agents produce considerable activation, the extent of activation depending upon the activity

of the enzyme preparation. Thus preparations having low activity are activated to a greater extent than the highly active ones. A reverse effect is observed with oxidizing agents which inhibit preparations of high activity to a greater extent. These results can be explained by the sulphydryl nature of the enzyme. Thus while the action of oxidizing agents consists in the oxidation of —SH to inactive —S—S— groups, a reverse effect is brought about by reducing agents. It may be further surmised that the degree of activation of the enzyme depends on the number of thiol groups present in the reduced state. The relative ease with which the enzyme is influenced by these various reagents shows that the active thiol groups of the enzyme protein are readily available for oxidation-reduction purposes.

Effect of some thiol- and amino-group inhibitors. The effect of some of these reagents on the activity of the enzyme is shown in Table 7. Inhibition is found to occur in all the cases. The inhibitory effects of alloxan, maleic acid and arsenite were completely abolished by the presence of 2 mg./ml. of cysteine in the reaction mixture. These substances thus seem to exert their effect by affecting the —SH group of the enzyme protein. Inhibition by formaldehyde and nitrous acid suggests further that amino groups may also be necessary for the activity of the enzyme.

Table 6. *Effect of oxidizing and reducing agents*

(0·1 ml. of the enzyme + buffer at pH 6 + the indicated substance in a final concentration of 0·001 M incubated at 30° for 15 min. After addition of substrate, incubation was continued at 37° for 30 min. with active enzyme preparations (E_1 and E_3), and for 60 min. in the case of weak enzyme preparation (E_2). Enzyme activity units are given in brackets. These units are expressed in terms of μg. of inorganic phosphorus liberated in 30 min. in the absence of activator.)

Substance	Activation (+) or inhibition (−) (%) E_1 (56 units)	E_2 (17 units)	E_3 (133 units)
Oxidizing agents			
Ferricyanide	−87·5	−45·5	—
Hydrogen peroxide	−45	−29	—
Ferric chloride (0·01 M)	−100	−44	—
Reducing agents			
Ascorbic acid	+175	+508	+90
Cysteine	+97·5	+376	—
Thioglycollic acid	+107·5	+596	+92
Sodium cyanide	−47·5	−29	−32

Table 7. *Effect of some thiol- and amino-group inhibitors*

(0·1 ml. of the enzyme + buffer at pH 6 + indicated substance were incubated at 30° for 15 min. After addition of substrate, incubation was continued at 37° for 30 min.)

Substance	Molarity	Inhibition (%)
Alloxan	0·001	64
Maleic acid	0·001	42
Arsenite	0·001	38
Formaldehyde	0·01	38
Sodium nitrite	0·01	55

DISCUSSION

From the activation and inhibition studies it would appear that the enzyme is inhibited by heavy-metal ions, oxidizing agents and thiol inhibitors. Iodoacetic acid has been reported to be a strong inhibitor for the enzyme (Norberg, 1950). Further, the enzyme is activated to an appreciable extent by reducing agents. Put together, these facts point towards the indispensability of sulphydryl groups for the activity of the enzyme. No satisfactory metal-ion activator has so far been found for the enzyme. Furthermore the enzyme is unaffected by dialysis, and therefore no dialysable coenzyme seems to be necessary for enzyme activity.

It will be interesting at this stage to compare the properties of phosphoprotein phosphatase with that of the acid phosphomonoesterase occurring in ox spleen (Davies, 1934). Both exert their maximum activity at about pH 6 and have their maximum stability at pH 5–6. Magnesium has no action on either of them. However, although the former appears to be a sulphydryl enzyme, none of the phosphomonoesterases has so far been found to require sulphydryl groups for its activity. Moreover, it has been found possible in the present investigation to separate the two enzymes from each other to a certain extent (Table 2). Finally, the inability of our purified enzyme preparation to dephosphorylate phosphopeptone to any appreciable extent rules out the possibility of the enzyme being identical with any of the phosphomonoesterases which, as is known, hydrolyse phosphopeptone but not casein (Schmidt & Thannhauser, 1943; Perlmann, 1952). It is obvious, however, that the action of this enzyme on other phosphorus esters has to be studied before any conclusion can be drawn as regards its absolute specificity. Also the exact physiological role of the enzyme remains to be investigated. A preliminary step in this direction has been made by Norberg (1951).

SUMMARY

1. Phosphoprotein phosphatase has been shown, for the first time, to be present in cattle tissues.

2. A procedure has been worked out for the partial purification of the enzyme from ox spleen. Using a combination of ammonium sulphate- and acetone-fractionation procedures, a 200-fold purifi-

cation of the enzyme with an over-all yield of 20% was effected.

3. A purified preparation of the enzyme attacked all three phosphoproteins studied, but had no appreciable action on glycerophosphate or casein phosphopeptone. The enzyme is hence a true phosphoprotein phosphatase, distinct from the acid phosphomonoesterase.

4. Optimum enzyme activity was found at pH 6·0 and at a substrate concentration corresponding to about 10 μmoles/ml. of casein phosphorus with 0·001 M thioglycollic acid as activator.

5. From the activation and inhibition studies it is deduced that sulphydryl and amino groups are essential for the activity of the enzyme. However the enzyme requires no dialysable coenzyme for its activity.

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The Synthesis of Coenzyme A by *Lactobacillus arabinosus* 17–5

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Washed, glycolysing suspensions of haemolytic streptococci cause a disappearance of pantothenate from the suspending medium (McIlwain & Hughes, 1944, 1945). As most of the pantothenate in animal and bacterial tissues exists in the form of coenzyme A (CoA) (Novelli, Kaplan & Lipmann, 1948) it seemed likely that the synthesis of CoA was the process responsible for the disappearance of pantothenate. Washed suspensions of *Lactobacillus arabinosus* 17–5 were found to take up pantothenate. The fate of pantothenate was studied in this organism because it requires pantothenate for growth and, unlike streptococci (McIlwain & Hughes, 1945) and *Escherichia coli* (Maas, 1952), it does not decompose pantothenate.

Pantothenate was added to washed suspensions and its disappearance was measured by microbiological assay. A major fraction of this pantothenate was converted into coenzyme A, which was determined by enzymic assay.

The part of this work in which cystine was shown to be essential for coenzyme A synthesis was communicated to the 2nd International Congress of Biochemistry (Pierpoint & Hughes, 1952).

EXPERIMENTAL

Materials. The acetylating enzymes required for the estimation of coenzyme A according to Handschumacher, Mueller & Strong (1950) and Lipmann, Kaplan, Novelli, Tuttle & Guirard (1947) were obtained from the livers of decapitated pigeons. The 4-aminoazobenzene was a chromatographically pure specimen prepared and given by Dr J. A. Miller (University of Wisconsin). Adenosine triphosphate (ATP) was prepared by the method of LePage (1949): the chromatographic procedure, described by Eggleston & Hems (1952), showed that 90% of the phosphate of the sample was present in the form of ATP.

Transacetylase was obtained from a strain of *Esch. coli* (National Collection of Type Cultures, strain 86) grown in 10 l. batches of medium containing 0·5% (w/v) Pronutrin (a commercial casein digest marketed by Herts Pharmaceuticals Ltd., Welwyn), 1% (w/v) glucose, 0·4% (v/v) boiled yeast extract, 0·4% (w/v) KH_2PO_4, 0·25% (v/v) salts mixture B of Barton-Wright (1946), 10% (v/v) tap water, pH 7·0. The cells were dried over P_2O_5 *in vacuo* and the enzyme extracted according to Novelli & Lipmann (1950). Acetyl phosphate was prepared by the method of Lipmann & Tuttle (1944), and found to be 87% pure by phosphate analysis (Berenblum & Chain, 1938) and by using succinic anhydride as a standard in the hydroxamic acid reaction (Lipmann & Tuttle, 1945).

The samples of CoA used as standards were various preparations from dog liver (Kaplan & Lipmann, 1948). Others were obtained commercially from Armour Laboratories, and had an activity of 10 Lipmann units/mg. Casein hydrolysate was prepared according to Barton-Wright (1946). Pantetheine was prepared and given by Drs Baddiley & Thain (1952).

Organisms. Lb. arabinosus 17–5 was grown in the semi-synthetic medium described by Barton-Wright (1946) for the assay of pantothenate. Satisfactory yields of CoA-deficient organisms (20 mg. dry wt./100 ml.) were obtained after 16–18 hr. growth at an initial pantothenate concentration of 1×10^{-7} M. Up to 5 times higher yields were obtained by increasing the pantothenate concentration to 1×10^{-2} M, but the cells contained more CoA and synthesized less on further incubation (Table 1). The cells were harvested by centrifugation, washed in 0·9% (w/v) NaCl and suspended in a solution containing 0·05 M glucose and 0·05 M phosphate buffer pH 6·5, in the saline of Krebs & Henseleit (1932).

Estimations of coenzyme A. To extract the intracellular CoA, the centrifuged cells were suspended in 0·5 ml. of water in stoppered tubes, and placed in a boiling-water bath for 10 min.

The assay routinely used was that of Handschumacher *et al.* (1950), which is based on the CoA-dependent acetylation of 4-aminoazobenzene. In addition the method of Kaplan & Lipmann (1948), in which sulphanilamide acts as acetyl acceptor, was used. This served to check the results obtained by the other method and also to calibrate freshly prepared samples of CoA in terms of the generally accepted Lipmann units.

Pantetheine has CoA activity when unfractionated pigeon-liver acetone powder is used as acetylating enzyme, owing to the presence of CoA-synthesizing enzymes (Govier & Gibbons, 1952). In order to check the results obtained when pantetheine was present in the incubation mixtures, estimations were made by the arsenolysis method of Stadtman, Novelli & Lipmann (1951) which is unaffected by pantetheine.

As shown in Table 2, known amounts of CoA added to cell suspensions were satisfactorily recovered by the method used. Addition of 0·1% (w/v) Na_2S, $9H_2O$ to the suspension during the extraction did not affect the recovery.

Estimations of pantothenate. Pantothenate was estimated essentially as described by Barton-Wright (1946), by measuring turbidimetrically the growth response of *Lb. arabinosus* 17–5. Combined pantothenate was estimated by assaying before and after treatment with a commercial sample of intestinal phosphatase and chicken-liver enzyme prepared and used in the manner described by Novelli & Schmetz (1951).

Conditions of incubation. Samples (1 ml.) of the cell suspensions were put into a series of 10 ml. centrifuge tubes, together with solutions of the substances to be tested, which had been adjusted to pH 6·5. The total volumes were made up with distilled water to convenient values, generally 2 ml., and the tubes incubated for periods up to 60 min. Unless otherwise stated all incubations were at 37°. Manometric experiments, in which glycolysis was followed by liberation of CO_2 from bicarbonate, showed that under these conditions lactic acid production continued linearly for approx. 90 min. There was no difference in the rate of glycolysis of pantothenate-deficient cells when incubated with and without pantothenate and cystine. Metabolic activities were stopped by cooling the tubes in freezing mixture. The cells were collected by centrifugation, the mother liquors decanted off and the tubes wiped dry with filter paper. When pantetheine was present in the incubated mixture, the cells were washed with fresh saline containing glucose and phosphate to remove surplus pantetheine. Glucose saline was used, as cells tend to lose coenzyme A when incubated in absence of glucose.

Table 1. *The effect of the concentration of pantothenate in the growth medium on the synthesis of coenzyme A by washed suspensions*

(Cells were collected after 16–17 hr. growth, and incubated under the standard conditions described in the text.

Concentration of pantothenate in the growth medium (M)	Yield of cells (mg. dry wt./100 ml.)	CoA content of cells (Lipmann units/g. dry wt.)	
		Before incubation	After incubation
2×10^{-7}	29	30	750
10^{-4}	100	350	640
5×10^{-5}	105	700	800
10^{-2}	90	600	630

Table 2. *Recovery of coenzyme A*

(3·0 Lipmann units were added to suspensions of 4 mg. of cells of *Lactobacillus arabinosus*, heated and then assayed as described in the text.)

Heat treatment	% Recovery (Average of duplicate tubes)	
	Expt. 1	Expt. 2
80° for 15 min.	100	107
100° for 5 min.	93	104
100° for 10 min.	106	105
100° for 15 min.	105	102

RESULTS

The effect of constituents of the growth medium upon coenzyme A synthesis by washed suspensions. Cells grown in the presence of 1×10^{-7} M pantothenate contained from 15 to 100 Lipmann units of CoA/g. dry weight compared with 600–900 units/g. dry weight in cells grown in an excess of pantothenate (10^{-3} M). Washed suspensions of the deficient cells synthesized an extra 50–100 units CoA/g. dry weight when incubated in the glucose-saline mixture with added pantothenate. This meant that the cells contained about 200 units CoA/g. dry weight, which is considerably less than the amount found in cells grown with adequate pantothenate, and it suggested that some other constituent of the growth medium was essential for maximum CoA synthesis. As casein hydrolysate increases the uptake of pantothenate by haemolytic streptococci (McIlwain & Hughes, 1945), its effect on CoA synthesis was tested. It increased both the rate of synthesis and the amount of CoA synthesized (Table 3). When synthetic mixtures of amino acids and single amino acids were tested for their ability to replace the casein hydrolysate, only cystine and cysteine were found to be

effective (Table 3). There was no additional increase in the amount of CoA synthesized when mixtures of amino acids or casein hydrolysate were incubated together with cystine. The effect of casein hydrolysate on the uptake of pantothenate found by McIlwain & Hughes was probably due to cystine.

The washed suspensions did not synthesize CoA when glucose was absent, indicating that CoA synthesis, like cozymase synthesis (Hughes & Williamson, 1952), depends on an energy supply. No other energy source was tested.

The omission of Mg^{2+} considerably decreased the amount of CoA synthesized (Table 4). This recalls a similar effect on the uptake of pantothenate (McIlwain & Hughes, 1945) and could be explained on the basis of the recent findings that at least one of the phosphorylating steps in CoA synthesis in liver depends on the presence of Mg^{2+} (Levintow & Novelli, 1952). Glutamic acid, which has been reported to stimulate the growth-promoting properties of pantothenate (King, Fels & Cheldelin, 1949) and to reverse the inhibitory effects of phenylpantothenone (Woolley, 1946) had no effect on CoA synthesis. Similarly, uracil, guanine, and adenine were without effect.

The effect of pantothenate and cystine concentration. The amount of CoA synthesized in glucose-saline containing 2×10^{-4}M cystine increased when the concentration of pantothenate was raised from 1×10^{-6} to 2×10^{-5}M (Fig. 1); there was no further increase in concentrations up to 1×10^{-3}M. The amount varied in a similar fashion when the panthothenate concentration was kept constant and the cystine concentration was varied from 1×10^{-6} to 5×10^{-5}M (Fig. 1). The amount formed was maximal when the concentrations of both cystine and pantothenate were greater than 2×10^{-5}M. For testing the effect of other conditions on CoA synthesis, the concentration of pantothenate and of cystine was generally 2×10^{-4}M. Under these conditions CoA synthesis continued linearly for 60 min. (Fig. 2), and ceased when the cells contained approximately 600–800 Lipmann units.

Table 3. *The effect of amino acids on coenzyme A synthesis*

(Each tube contained: 8·5 mg. dry wt. cells suspended in phosphate-saline containing glucose; $2{\cdot}5 \times 10^{-4}$M pantothenate; the amino acids indicated below in a final volume of 2·0 ml.)

Amino acid source added (final concentration)	CoA synthesized (Lipmann units/g. dry wt.)
None	109
·5% (v/v) Casein hydrolysate	360
10^{-4}M Cystine	370
10^{-4}M Cystine; 5% (v/v) casein hydrolysate	350

Table 4. *The effect of* Mg *on synthesis*

(Each tube contained: 10 mg. cells suspended in the phosphate-saline containing glucose without Mg; $2{\cdot}5 \times 10^{-5}$M pantothenate; $1{\cdot}5 \times 10^{-4}$M cystine, and the additions listed below, in a final volume of 2·0 ml.)

Additions to mixture	CoA content of cells (Lipmann units/g. dry wt.) Before incubation	After incubation
None	50	265
$7{\cdot}7 \times 10^{-4}$M-$MgSO_4$	50	470

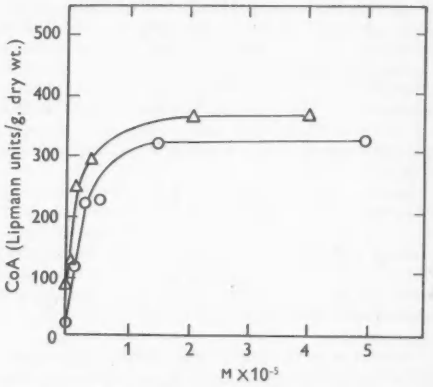

Fig. 1. Effect of pantothenate and cystine concentration on coenzyme A synthesis. Tubes contained 2 ml. of phosphate-saline containing glucose together with the following additions: ○—○, 8·4 mg. of cells (batch A) in 2×10^{-4}M pantothenate and varying amounts of cystine; △—△, 13 mg. of cells (batch B) in 2×10^{-4}M cystine and varying amounts of pantothenate.

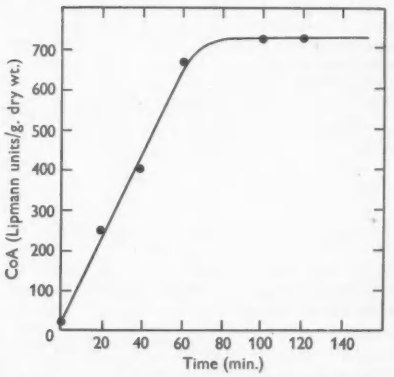

Fig. 2. Time curve of coenzyme A synthesis. Washed pantothenate-deficient cells (9·7 mg. dry wt.) were suspended in 2 ml. of the phosphate-saline containing glucose and 2×10^{-4}M cystine and 2×10^{-4}M pantothenate.

Table 5. *The effect of the age of cells on their ability to synthesize coenzyme A*

(For experimental details see text.)

Expt.	Age of cells (hr.)	Stage of growth	CoA content of cells (Lipmann units/g. dry wt.) Before incubation	After incubation	CoA synthesized
1	10	Beginning of log phase	190	300	110
	16	Log phase	240	440	200
	20	Stationary phase	260	530	270
	32	Stationary phase	0	300	300
2	9	Beginning of log phase	130	280	150
	12	Log phase	160	780	600
	18	End of log phase	135	685	550
3	17	End of log phase	230	480	250
	41	Stationary phase	42	272	230

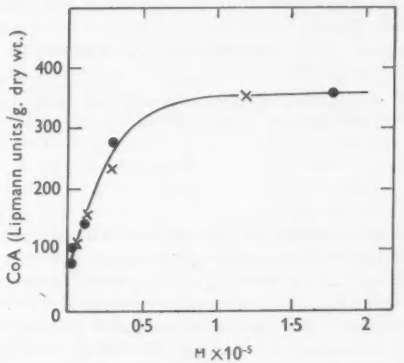

Fig. 3. Synthesis of coenzyme A from pantetheine. Deficient cells (8·0 mg. dry wt.) were suspended in the phosphate-saline containing glucose (final volume 1·8 ml.) with various amounts of cystine (●) or pantetheine (×). Those tubes containing cystine also contained 2×10^{-5} M pantothenate.

Table 6. *Comparison of coenzyme A estimations by arsenolysis and acetylation tests*

(Deficient cells (10 mg. dry wt.) were incubated in the phosphate-saline mixture with 10^{-4} M pantetheine, the cells collected, washed in fresh saline, and the CoA extracted and estimated.)

Method of estimation	CoA (Lipmann units/g. dry wt.) Before incubation	After incubation	Synthesized
Acetylation	100	600	500
Arsenolysis	400	950	550

The effect of age of cells upon coenzyme A synthesis. To test the effect of the age of cells on CoA synthesis, samples from large batches (1000 ml.) of the pantothenate-deficient culture were examined at intervals for their ability to synthesize CoA, as already described. The CoA content of the cells was approximately constant during the log phase of growth and decreased during the stationary phase, until almost all of the CoA had been lost (Table 5). The ability of the cells to synthesize was lowest at the beginning of the log phase, increased continuously during the log phase, and remained constant during the stationary phase (Table 5). This is in contrast to cozymase synthesis by this organism, which is greatest in the younger cells and decreases rapidly once the cells have ceased growing (Hughes & Williamson, 1952).

The effect of compounds related to cystine and pantothenate on coenzyme A synthesis. Cysteine and cystine were equally effective in increasing the rate and amount of coenzyme A synthesis. The amount of CoA synthesized by addition of an equivalent amount of SH-glutathione was only about 15% of that formed from cystine. It is uncertain whether this small effect of glutathione is due to contamination with cystine, or to slow hydrolysis. No effect was found when cystine was replaced by β-mercaptoethylamine, *N*-β-alanyl-β-mercaptoethylamine, taurine, cysteic acid, homocystine, methionine, *S*-methylcystine, thioglycollic acid, thioacetamide or ethanethiol.

Pantetheine completely replaced both pantothenate and cystine (Fig. 3), indicating that the role of cystine is to participate in the formation of the β-mercaptoethylamine residue of CoA. This result was confirmed by using the arsenolysis method of estimating CoA, which is not affected by pantetheine. A comparison of the two methods of estimation showed that although they gave similar values for the amount of CoA synthesized by a batch of organisms, the values obtained by the arsenolysis method for the total CoA content of the organisms were larger than those from the acetylation method (Table 6). This may be due to some material extracted from the cells which catalyses the non-enzymic decomposition of acetyl phosphate.

Effect of cystine on pantothenate uptake. When washed suspensions of deficient cells were incubated with amounts of pantothenate and cystine which did not limit synthesis, the ratios of pantothenate taken

Table 7. *The effect of cystine on the uptake of pantothenate*

(Washed cells were incubated in glucose-saline with pantothenate and cystine, as indicated below. After the incubation the cells were collected by centrifugation and washed in 0·9% saline. The CoA content of the cells, and the pantothenate content of the mother liquor were estimated.)

Expt.	Dry weight of cells/tube (mg.)	Cystine added (M)	Pantothenate Added (μg.)	Pantothenate Recovered (μg.)	Pantothenate Uptake (μg.)	CoA synthesized (Lipmann units/tube)	Pantothenate uptake (μg.) / CoA formed (Lipmann units)
1	13·8	$1·5 \times 10^{-3}$	2·85	1·40	1·45	2·0	0·73
		None	2·85	1·46	1·39	0·25	—
2	12	5×10^{-5}	2·93	1·22	1·7	3·3	0·55
		None	2·93	1·54	1·38	0·62	—
3	9·8	5×10^{-5}	6·8	3·5	3·5	4·3	0·82
		None	6·8	3·7	3·1	1·82	—

Table 8. *Pantothenate uptake in the absence of cystine*

(Deficient cells (15 mg. dry wt.) were incubated for 80 min. at 37° in 2 ml. of the phosphate-saline mixture, containing glucose and the additions described below. After incubation the cells were removed by centrifugation, washed twice in 5 ml. saline, and the pantothenate was estimated in the mother liquor and cell extracts.)

Additions	Pantothenate taken up by the cells (μm-moles)	CoA synthesized (μm-moles)	Pantothenate in extract (μm-moles): Before incubation with phosphatase	After incubation with phosphatase	After incubation with phosphatase + chicken liver
None	—	—	—	0·5	4·0
Pantothenate ($2·5 \times 10^{-5}$ M)	30	—	1·0	15	25
Pantothenate ($2·5 \times 10^{-5}$ M); cystine (5×10^{-5} M)	27	19·2	—	4	24

up/CoA formed (Table 7) agreed well with values of 0·65–0·71 μg. pantothenate/Lipmann unit reported for the pantothenate content of CoA (Lipmann *et al.* 1947; Beinert *et al.* 1953). Hence most, if not all, of the pantothenate taken up under these conditions is converted into CoA.

The amount of pantothenate which was taken up from the incubation medium was independent of the presence of cystine (Table 7). Thus, although cystine is essential for the conversion of pantothenate into pantetheine, and therefore to the formation of CoA, pantothenate may be bound in its absence. In an attempt to study the properties of the bound form, cells were grown in limiting amounts of cystine (10^{-5} M) and an excess of pantothenate (1×10^{-2}–1×10^{-3} M). However, the cells contained large amounts of free pantothenate, which were slowly released on washing with saline. Even after seven washings with 0·9% saline, pantothenate was still present in the supernatant, in quantities which were large compared to those of the bound form. This made a study of the bound form impossible.

More suitable material was obtained when growing cells were replaced by washed suspensions of pantothenate-deficient cells. These were incubated with glucose and pantothenate, in the absence of cystine, until 60–80% of the added pantothenate (50 μm-moles) had been taken up (60–80 min.). The cells were then separated and washed three times with saline, no pantothenate being detectable in the final washing. The cells were suspended in water and heated as described for the extraction of CoA. The extract contained no free pantothenate. When incubated with alkaline phosphatase (Novelli & Schmetz, 1951), 60–75% of the pantothenate that had been taken up by the cells was released (Table 8). If the incubation was carried out with phosphatase and chicken-liver enzyme 90–100% could be recovered.

When this was repeated with cells that had been incubated with glucose, pantothenate and cystine, only 10–20% of the growth-supporting activity of the bound pantothenate could be detected after incubation with phosphatase. This is probably due to the formation of pantetheine, which has about 10–15% of the ability of pantothenate to support the growth of *Lb. arabinosus* under the conditions used (McRorie & Williams, 1951), and which is formed from CoA on incubation with phosphatase (Brown & Snell, 1952). Incubation with phosphatase plus chicken-liver enzyme gave complete recovery (90–100%).

DISCUSSION

The route of coenzyme A synthesis in animal tissues is taken to be (see Levintow & Novelli, 1952):

(1) Pantothenate → pantetheine.

(2) Pantetheine + ATP → pantetheine 4-phosphate + ADP.

(3) Pantetheine 4-phosphate + ATP → pantetheine-adenosine nucleotide + pyrophosphate.

(4) Pantetheine-adenosine nucleotide + ATP → CoA + ADP.

As for the synthesis of CoA by bacteria, Craig & Snell (1951) suggest that (1) occurs at least in those bacteria in which pantetheine has greater growth-promoting activities than pantothenate. Even in organisms like *Lb. arabinosus*, which grow better on pantothenate than on pantetheine, this reaction may be a step in the synthesis of CoA. This is suggested by the fact that washed suspensions of the organisms utilize pantetheine as effectively as pantothenate plus cystine.

That β-mercaptoethylamine does not replace cystine, either for the growth of *Lb. arabinosus* or for the synthesis of CoA by washed suspensions, argues against the view that β-mercaptoethylamine is an intermediate in CoA synthesis. Experiments to be published in collaboration with Drs Baddiley and Mathias (Lister Institute) show that *N*-pantothenyl cysteine does not support either growth or the synthesis of CoA in *Lb. arabinosus*, or the growth of *Lb. helveticus* and *Proteus morganii*. However, since these papers have been prepared Brown & Snell (1953) report similar findings, but have shown that *N*-pantothenylcysteine is active as a growth factor for *Acetobacter suboxydans* and that it is probably a precursor of pantetheine in this organism.

The synthesis of CoA may not be the only reaction of pantothenate under the test conditions, as pantothenate disappears when cells are incubated in absence of cystine. The evidence so far available (release of pantothenate on addition of intestinal phosphatase) suggests that pantothenate undergoes phosphorylation in the absence of cystine. Whether this reaction occurs in the presence of cystine is uncertain.

SUMMARY

1. Glycolysing, washed suspensions of *Lactobacillus arabinosus* 17–5 grown on a medium deficient in pantothenate can synthesize 50–100 Lipmann units of coenzyme A/g. dry wt. from pantothenate. The addition of cystine increases the amounts of coenzyme A which can be synthesized to 600–800 Lipmann units/g. dry wt. The amounts of coenzyme A synthesized are related to the initial coenzyme A content of the cells; the most deficient cells synthesize most coenzyme A. Glucose and magnesium ions are essential for the synthesis.

2. Panthetheine can replace pantothenate and cystine. Cystine is presumably required for the formation of the β-mercaptoethylamine residue of panthetheine. It can be replaced by cysteine and partially by glutathione, but not by β-mercaptoethylamine, taurine, cysteic acid, homocystine, methionine, *S*-methylcystine, thioglycollic acid, thioacetamide and ethanethiol.

3. The ability of the cells to synthesize coenzyme A is smallest in cells harvested at the beginning of the log phase of growth, increases continuously during the log phase and remains constant for at least 42 hr. after the cells have ceased to grow.

4. Pantothenate is removed by the cells in the absence of cystine, and reappears on treatment with alkaline phosphatase. This suggests that pantothenate is phosphorylated in the absence of cystine.

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The Mechanism of β-Amylase Action

1. 'MULTICHAIN' ACTION ON AMYLOSE

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Amylose, the constituent of starch which consists essentially of unbranched glucosidic chains, is hydrolysed by β-amylase in stepwise fashion starting from the non-reducing end. The sole fission products are maltose and continually shortening amylose chains.

It was concluded by Swanson (1948) that the enzyme, having started the attack on one chain, completes its hydrolysis before beginning to attack another chain. The experimental evidence included the absence of short-chain fission products in dialysates of maize amylose undergoing β-amylolysis, and the unchanging pattern of its iodine coloration spectrograms. Simultaneously, Cleveland & Kerr (1948) came to the same conclusion (see also Kerr, 1949; Kerr & Cleveland, 1951). In these cases, maize amylose was partly hydrolysed and the residual polysaccharide was recovered and examined by various methods (viscosity, iodine coloration, osmotic pressure, ferricyanide number, etc.). The results indicated that it possessed a molecular weight approximating to that of the original amylose. The fission products, examined by paper chromatography, did not contain sugars of greater chain length than the expected maltose. Since amylose must consist of chains of widely varying lengths, it was inferred that the shorter ones are hydrolysed preferentially.

This hypothesis of amylase action, which has been described as the 'single chain' mechanism, has received wide acceptance but has been criticized by Hopkins & Jelinek (1949) and by Bourne & Whelan (1950), whose observations favour the 'multichain' hypothesis, namely that the enzyme attacks chains of all lengths at random and having completed a single fission, does not necessarily continue to attack the same chain.

Meyer, Bernfeld & Press (1940) had earlier concluded that the smaller molecules in maize amylose served to protect the larger ones from retrogradation, that they were attacked first by β-amylase, and that their removal in the earlier stages of hydrolysis facilitated retrogradation or, at least, ageing of the remainder.

French, Levine, Pazur & Norberg (1950), using amyloheptaose prepared from a Schardinger dextrin (cycloheptaose), obtained evidence that under conditions favourable to enzyme action, no pentaose is present in partly hydrolysed reaction mixtures. This observation supports the 'single chain' theory. On the other hand, French, Knapp & Pazur (1950) found that if the enzyme was made to act under certain unfavourable conditions (pH 10, or 70°), pentaose was formed, thus suggesting that the two mechanisms were liable to operate according to circumstances.

A review of the experimental evidence led the present authors to the opinion that no conclusion was yet justified and that the matter needed investigation by new methods. It was deemed desirable to prepare specimens of amylose and of its fission products with long and short chains, respectively, and to determine these chain lengths and the Michaelis constants of the specimens with β-amylase. These data would show whether the enzyme exhibited any marked differences in affinity for these products. As their iodine spectrograms would be characteristic and different, it should also be possible to follow their respective rates of disappearance when hydrolysed in mixtures.

EXPERIMENTAL

Materials

Amylose. This was prepared from potato starch as described by Hopkins & Jelinek (1948).

β-Amylase. This was prepared from soya beans as described by Newton, Hixon & Naylor (1943).

Amylose dextrins. Potato amylose (0·63%, w/v) was maintained at 80° in the presence of 0·1 N-HCl, and after 1 and 5 hr., respectively, a quantity was neutralized. No retrogradation had occurred.

Dextrin B. This was obtained from the 1 hr. hydrolysate by heating to 85° with excess of *n*-butanol, cooling slowly at first and finally in a refrigerator at 3°. A precipitate appeared at the last stage only. This was centrifuged, dissolved and twice reprecipitated in the same way. It gave a blue iodine coloration. Yield, 24·2%.

Dextrins R. These were obtained from the 5 hr. hydrolysate which was cooled slowly with excess *cyclo*hexanol; the small precipitate which formed was removed, and the filtrate evaporated to half volume and precipitated with 3·5 vol. 95% (v/v) ethanol. The precipitate was dissolved and reprecipitated, washed, and dried over $CaCl_2$ *in vacuo*. It gave a red iodine reaction. Three preparations were made. Yield, R_1 29·7%; R_2 and R_3, about 30%. Successive

dialyses of R_1 into large volumes of still water indicated very little difference in composition between the portions found outside and inside the membrane. R_2 and R_3 were thoroughly dialysed through cellophan before use.

Reducing powers. These were determined by the method of Blom & Rosted (1947). The Somogyi (1945) method was also used but gave somewhat lower results. Unless very minute quantities had to be measured, the Blom & Rosted method was preferred. The R_m value is the reducing power as the percentage of the reducing power of the theoretically obtainable maltose.

Periodate determinations. These were made by the method of Potter & Hassid (1948), slightly modified.

Absorption values. These were determined as described by McCready & Hassid (1943), using a Spekker absorptiometer with a 1 cm. cell. For dextrins B and R_1 (Table 1) and the experiment (Table 6) in which they were used together, the readings were made on a Coleman Universal Spectrophotometer. The numerical values of the readings, calculated for a 1 cm. cell, were almost identical with the corresponding readings on the Spekker absorptiometer.

Michaelis constants (K_m). These were determined as described by Hopkins, Jelinek & Harrison (1948), except that reducing powers were measured by the methods of Blom & Rosted (1947), and of Somogyi (1945).

RESULTS

The absorption values and chain lengths of the materials used are summarized in Table 1, and the Michaelis constants of four different preparations of amylose and of two fission products are given in Table 2. The value of K_m, expressed in molar concentration, did not vary much among the six preparations, while the maximum velocity varied even less. Data from which values of K_m were calculated are given in Table 3.

The soya bean β-amylase readily hydrolysed amylose at pH 4·8 to R_m 90 (Table 4), and must therefore have contained the Z-factor of Peat, Pirt & Whelan (1952).

In the hydrolysis of the mixture of amylose and dextrin R_3 (Table 5), absorption values lost over the period 0–30 min. are almost identical with those found for 1 mg. of dextrin R_3/100 ml. (Table 1). Thus, in effect, attainment of R_m 48 in this reaction mixture has been accompanied by the removal of 1 mg. of dextrin R_3/2 ml. (the volume withdrawn) or 50 mg./100 ml. of reaction mixture. Actually, rather less than this amount must have been attacked, for a small contribution to the change in absorption values would be made by the amylose when undergoing fission.

With the mixture of dextrins B and R_1 (Table 6) the loss over the period 0–45 min. is of material of similar character in its iodine coloration to dextrin R_1.

Table 1. *Absorption values and chain lengths*

(The solutions contained 1 mg. polysaccharide, 10 mg. I_2 and 100 mg. KI in 100 ml. and were examined in a 1 cm. cell.)

	Chain length calculated from		Absorptiometer readings at (mμ.)						
	Blom & Rosted (1947)	Periodate oxidation	680	630	600	550	500	490	470
Amylose	—	Approx. 640	382	—	338	223	—	130	120
Dextrin B	Approx. 50	—	287	318	304	226	141	133	122
Dextrin R_1	12·5	—	21	42	65	112	144	141	131
Dextrin R_2	19·6	20	25	—	47	—	—	132	117
Dextrin R_3	20·9	22·5	30	—	55	—	—	154	140

Table 2. *Michaelis constants and maximum velocities of β-amylolysis of potato amylose and its short-chain fission products*

(Enzyme action carried out at pH 5·8 and 30°.)

	Mean chain length calculated from			Michaelis constant (K_m)		Maximum velocity (V) (mg. maltose/ 100 ml./ min.)
		Reducing power				
	Periodate oxidation	Blom & Rosted (1947)	Somogyi (1945)	(%)	M × 10^{-5}	
Amylose 1	360	—	—	0·32	5·5	—
Amylose 2	300	—	—	0·32	6·7	—
Amylose 3	450	—	—	0·7	9·0	2·5
Amylose 4	660	—	—	0·85	8·0	2·5
Short-chain fission product, R_2	20	19·6	22	0·0156	4·8	3·0
Short-chain fission product, R_3	22·5	20·9	—	0·0163	4·7	2·4

Table 3. *Velocity of β-amylolysis of amylose and amylose dextrins*

(Reaction mixtures contained 0·4 mg. soya bean β-amylase, 1 ml. McIlvaine buffer, pH 5·8, for each 100 ml. substrate. Temperature, 30°.)

	Substrate concn. (mg./100 ml.)	Time (min.)	Maltose formed (mg./100 ml.)	K_m (%)	Maximum velocity (mg./100 ml./min.)
Amylose 3	44	17·8	2·8	0·70	2·5
	88	20·0	5·6	—	—
	177	22·4	11·2	—	—
	354	26·9	22·4	—	—
Amylose 4	51	22·7	3·25	0·85	2·5
	102	24·8	6·5	—	—
	205	27·1	13·0	—	—
	410	32·0	26·0	—	—
Dextrin R_2	23·5	5·5	9·9	0·0156	3·0
	47	8·8	19·8	—	—
	94	15·2	39·6	—	—
	188	28·3	79·2	—	—
	375	54·6	158·4	—	—
Dextrin R_3	50	11·8	21	0·0163	2·4
	100	20·5	42	—	—
	151	29·3	63	—	—
	201	38·0	84	—	—

Table 4. *Progress of action of soya bean β-amylase on amylose*

(Reaction mixture: amylose (0·5%) and soya bean β-amylase (2·0 mg./100 ml.) at pH 4·8 and 30°.)

Time (min.)	R_m
10	18
25	31·3
40	41·5
80	58·5
145	67
19 (hr.)	88

Table 5. *β-Amylolysis of a mixture of amylose and dextrin* R_3

(33·3 mg. Amylose 4, 63·8 mg. dextrin R_3, 0·5 ml. McIlvaine buffer solution, pH 5·8, and soya bean β-amylase. Total volume, 100 ml. Incubated at 30°. Samples (2 ml.) withdrawn at intervals into 10 mg. I_2 + 100 mg. KI and diluted to 100 ml.)

Time (min.)	R_m (%)	Absorption values at (mμ.) 680	600	490	470
0	6·4	294	300	272	250
15	36·3	293	274	148	133
30	55	266	242	101	91
Difference (0–30 min.)	48·6	28	58	171	159
60	61·6	249	222	89	84

Table 6. *β-Amylolysis of a mixture of dextrins* B *and* R_1

(The reaction mixture contained 208 mg. dextrin B, 182 mg. dextrin R_1, 16 ml. 0·02 N acetate buffer (pH 4·6) and 1 mg. soya bean β-amylase. Total volume 220 ml. Temp. 30°. Samples (1 or 2 ml.) withdrawn at intervals into 10 mg. I_2 and 100 mg. KI, and diluted to 100 ml. A further 2 mg. of enzyme were added after 125 min. and again after 185 min.)

Time (min.)	R_m (%)	Absorption values (calculated to 1 ml. withdrawals) at (mμ.) 680	630	500	490
0	10	288	336	252	243
45	27·1	273	305	171	165
Difference (0–45 min.)	17·1	15	31	81	78
180	55	135	151	89	85
Difference (45–180 min.)	27·9	138	154	82	80
240	85·5	12	15	9	9

DISCUSSION

The Michaelis constant (K_m) for amylose and soya bean β-amylase has been shown to vary (Hopkins *et al.* 1948) when expressed as a percentage, but it does not follow that any such variation occurs when calculated to molar concentration. The results in Table 2 suggest that the affinities of both normal amylose and short-chain fission products, expressed on a molar basis, are all of the same order. The maximum velocities of β-amylolysis of these substrates are also approximately the same. We may therefore conclude that there is no great disparity between the rates at which the enzyme splits maltose from long and short amylose chains. In any event, both kinds of chain are liable to simultaneous attack, and it seems that the same would hold for the chains of the varying lengths actually present in amylose.

Hopkins *et al.* (1948) concluded that the longer the chain the faster the β-amylase hydrolyses the amylose chain. One would expect the reaction to be of zero order, especially when a high concentration of substrate is employed, but these authors found that the rate declined continually, and the above conclusion was reached in order to explain this observation. However, a major factor in explanation of the decline in rate reported by Hopkins *et al.* (1948) and unknown at that time, is the marked check in hydrolysis as R_m 70 is approached (Peat *et al.* 1952). It is now apparent that, at this stage, the rate of the reaction is increasingly controlled by the activity of the Z-enzyme (Peat *et al.* 1952) and, perhaps, by traces of α-amylase.

Let us consider a reaction mixture containing two dextrins derived from amylose, consisting respectively of long and short chains. This is submitted to the action of β-amylase. If the affinity constants (molar) were the same and if diffusion did not influence one constituent more than the other, the proportion of the chains of each constituent to be attacked at the outset would be the same. If the 'single chain' mechanism operated, the enzyme would complete the hydrolysis of a chain, then attack a fresh chain chosen at random from the residue of unattacked chains. As this residue would consist of the same relative proportion of the two dextrins as did the original mixture, the proportions of the components to be attacked would remain unchanged. The two dextrins would disappear from the medium at rates proportional to their original molar concentrations, their relative concentrations remaining unaltered. The absorption values at all wavelengths would decline proportionally.

If, however, the 'multichain' mechanism operates, all chains, long and short, would be shortened by an equal number of maltose units in a given time. Soon the short-chain component would have arrived at chain length 7 and have ceased to give an iodine colour, whereas the other one would be comparatively unaffected in this respect. The polysaccharide disappearing would be iodine-reddening material, whereas the residue would give a series of absorption values corresponding to bluer material than the original. These latter changes are those which were observed (Tables 5 and 6).

This is more clearly seen when amylose itself was one constituent (Table 5), owing to the marked difference between the iodine spectrograms of the constituents, and the failure of amylose to change appreciably in this respect when the chains are slightly shortened. When amylose dextrin of chain length 50 (Table 6) was employed the result was less clear cut, but still convincing.

Even an advantage secured by short chains by virtue of diffusion rates could scarcely account for the difference between dextrins B and R_1 which had mean chain lengths 50 and 12, respectively. The reason why the short-chain constituent disappears before the other is that its chains, being shorter, are finished first.

In considering the results in Tables 5 and 6 it is not essential to postulate that the affinity of the enzyme for the long and short chains is identical. In Table 6 the whole of the dextrin R_1 originally present would be responsible for the following absorption values: 17·3 (680 mμ.), 34·6 (630 mμ.), 119 (500 mμ.), 116 (490 mμ.), these values being calculated from the data in Table 1 and the concentration of R_1 in the reaction mixture. The material removed in the first 45 min. is, from its absorption value, of the character of dextrin R_1, and is equivalent to about 66% of the total originally present. Since dextrin R_1 amounted to 47% of the total substrate we thus have about 31% of the substrate disappearing whilst only 17·1% of the total theoretical amount of maltose has made its appearance. This could not be satisfactorily accounted for by 'single chain' amylolysis which would require that these figures agree. But on the 'multichain' mechanism the figures are easily understood. Thus, the complete β-amylolysis of dextrin R_1 would raise its R_m value from 16 to 100, or, in the reaction mixture, from 7·5 to 47, an increase of 39·5. The 17·1 increase actually observed is equivalent to shortening the mean chain length of dextrin R_1 by $(12{\cdot}5-2) \times 17{\cdot}1/39{\cdot}5 = 4{\cdot}5$, i.e. from 12·5 to 8. Since not all of the 17·1 increase in R_m is derived from dextrin R_1, the chains of the latter would not have been shortened quite so much. This result is in much better agreement with the observed loss of absorption value than that deduced above on the 'single chain' hypothesis.

Our soya bean β-amylase exhibited slight α-amylase activity which was almost negligible at pH 4·6. Any action by α-amylase would, however,

help to change amylose and dextrin of chain length 50 into red-staining dextrins and to act in opposition to the evidence reported.

SUMMARY

1. The Michaelis constants of soya bean β-amylase with potato amylose and its short-chain fission products are of the same order. The enzyme will therefore exhibit no decided preference for either long or short chains.

2. The early disappearance of the short chains from a mixture of long- and short-chain polysaccharides is demonstrated and must be attributed to a 'multichain' mode of attack by the enzyme, the hydrolysis of short chains being soon completed because they are short.

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The Mechanism of β-Amylase Action

2. 'MULTICHAIN' ACTION ON AMYLOSE FISSION PRODUCTS

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The evidence respectively favouring the 'single chain' and 'multichain' theories of β-amylase action has been summarized in the previous paper (Hopkins & Jelinek, 1954). Particular mention must be made of the work of French, Levine, Pazur & Norberg (1950) who hydrolysed amyloheptaose with soya bean β-amylase and found only maltose and maltotriose among the fission products. Even at the stage of 55% hydrolysis, no pentasaccharide was found, and the 'single chain' hypothesis received support. A preliminary experiment, reported briefly by Hopkins & Jelinek (1949), encouraged investigation of the action of β-amylase on amylose dextrins of chain length 10–30, the progress of which could be followed by changes in the absorption values of the iodine colorations. Such dextrins, prepared from amylose by acid or α-amylolytic fission, are colourless with iodine if shorter than chain length 8 (Swanson, 1948), and change from red through purple to blue over the range 10–30. The absorption value at 600–680 mμ. (blue value) varies directly with chain length over this range (Bailey, Whelan & Peat, 1950). If an amylose dextrin of chain length 20 is hydrolysed by β-amylase by 'single chain' mechanism the absorption values at all wavelengths should vary inversely with increase in reducing power as percentage from R_m 10, the initial value, to approximately R_m 100 (the R_m value is the reducing power of the theoretically obtainable maltose). If, however, a 'multichain' mechanism operates, when all chains are reduced in length at equal rates, the absorption value will fall to zero by the time that the chain length has been shortened to 8 units (R_m 70) and even a little earlier when measured at 680 mμ. Moreover, the relationship would be linear over this range of chain length.

An additional method of testing for the mechanism by which amylose short-chain fission products are degraded by β-amylase employs chromato-

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graphy. A hexasaccharide undergoing hydrolysis clearly shows the simultaneous presence of maltose, tetrasaccharide and unchanged hexasaccharide.

Although this communication is concerned with short-chain dextrins as substrate, one experiment with amylose is also included in which the presence of short-chain fission products was demonstrated.

MATERIALS AND METHODS

Amylose. This was prepared from potato starch as described by Hopkins & Jelinek (1948).

Amylose dextrins. These were prepared by acting on 0·5% (w/v) amylose with α-amylase (*Bacillus subtilis*) at pH 6, until the iodine reaction was purple, red or achroic, respectively, and boiling to stop further action. In the case of red-staining dextrins (R_1, R_2), this was followed by evaporation to a thin syrup, then precipitation and reprecipitation with acetone. The product was far from homogeneous at this stage and was repeatedly dialysed through cellophan. This removed all dextrins of shorter chain length and even permitted the slow diffusion of some of the required dextrin itself. No dextrin could be precipitated from the purified product by thymol or *n*-butanol.

Dextrins P_1 and P_2 were prepared by cooling with *n*-butanol at the purple-staining stage to remove the longest chains, then cooling the clear liquid to 3° overnight and collecting the sediment. The latter was redissolved and reprecipitated three times by cooling the aqueous solution to 3° without *n*-butanol. At each successive cooling the absorption values at various wavelengths were read for both the supernatant liquid and the sediment. The pattern of these for the liquid corresponded to dextrins of shorter chain length than that for the sediment, but the difference between liquid and sediment in this respect diminished with successive coolings. An attempt to fractionate the final sediment, P_1, by once more cooling its solution gave fractions which were indistinguishable in their absorption values at the same concentration. P_2 was obtained from the previous supernatant liquid, purified by dialysis. Further treatment by cooling with *n*-butanol removed nothing from either P_1 or P_2.

Achroic dextrin. This, after boiling and cooling, was put directly through a charcoal-Celite column (170 × 34 mm.) as described by Whistler & Durso (1950) and by Bailey *et al.* (1950). Successive elutions by water, 5, 15, 20, 25 and 30% (all v/v) ethanol yielded glucose, maltose, tri-, tetra-, penta- and hexa-saccharide, respectively. The elution of a given constituent was continued until no further material came off the column. Each eluate was concentrated and precipitated with acetone. The higher saccharides were refractionated.

The trisaccharide (maltotriose) had $[\alpha]_D$ (at equilibrium), $+158° \pm 1°$ in water. The hexasaccharide (R_m 32–33) had $[\alpha]_D$ (at equilibrium) $+177° \pm 1°$ in water.

β-Amylase. A sample of β-amylase was prepared from soya beans as described by Newton, Hixon & Naylor (1943). For a specimen of crystalline sweet-potato β-amylase, we are indebted to Dr A. K. Balls.

Absorption values. These were determined as described by Hopkins & Jelinek (1954).

Reducing power. This was measured by the method of Blom & Rosted (1947).

Paper-partition chromatography. This was performed as described previously (Bird & Hopkins, 1954).

RESULTS

Action of β-amylase on the dextrins

Purple-staining dextrins P_1 and P_2 and red-staining dextrins R_1 and R_2 were hydrolysed with β-amylase as summarized in Figs. 1–4, in which the absorption value, expressed as a percentage of the original value at 680 mμ., is plotted against R_m. The graph in all cases was at first linear, and its initial slope, if maintained, would have intersected the horizontal axis at R_m 50–70. Crystalline β-amylase, acting at pH 3·6 on the dextrin P_1 (purple) in a very rapid reaction and on the dextrin P_2 (reddish purple) was clearly destroying iodine colouring power much faster than it was producing maltose. Changing the conditions to pH 6·8 and 32° did not affect this result. Soya bean β-amylase was more effective in this respect at pH 4·6 in a fast reaction than at pH 5·8 in a much slower reaction. Soya bean β-amylase, preheated to 60° in solution at its natural pH for 1 hr., behaved, in a slow reaction at pH 4·6, just as in Fig. 4 at pH 5·8.

The experiment on which Fig. 2 is based is considered more fully in a later section (Table 2). During the main part of the reaction, the percentage loss in absorption value greatly exceeded the percentage hydrolysis. Blue values (680 mμ.) fell faster than red. When the reaction reached the end of the first linear phase, about 10% of absorption value still remained.

Action of crystalline β-amylase on linear hexasaccharide

A solution of hexasaccharide (1·5%, w/v) was incubated with the enzyme at 21°. Withdrawals of 20 μl. were placed directly on the paper and the chromatograms were developed.

Figs. 5–7 show that with the progressive disappearance of hexasaccharide, tetrasaccharide concentration increased at first and then remained constant, while maltose concentration steadily increased. Only after the disappearance of hexasaccharide at about R_m 65–70 did the tetrasaccharide concentration diminish. Traces of maltotriose were formed from penta- or hepta-saccharides present in the original substrate. The hexasaccharide used for Fig. 7 was more contaminated with pentasaccharides than the others.

Action of crystalline β-amylase on maltotriose

A solution of maltotriose (0·4%, w/v) was incubated with the enzyme at 26°. After 4 days action at pH 3·6 and at pH 5·8, chromatograms showed that the triose had nearly disappeared, while maltose and glucose had appeared in corresponding amounts. Action went further at pH 5·8 than at 3·6, as expected.

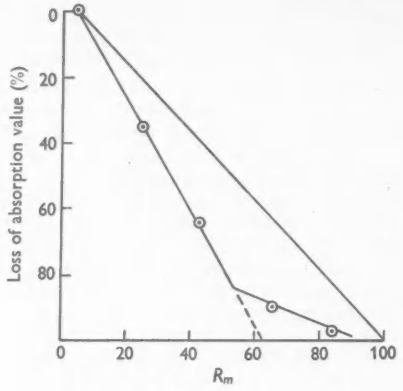

Fig. 1.

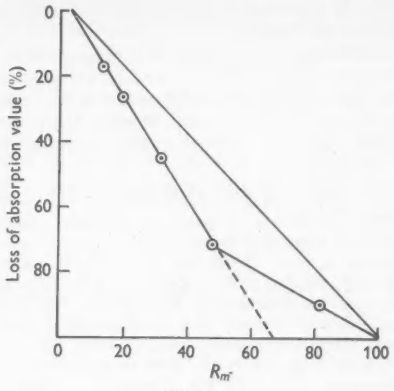

Fig. 2.

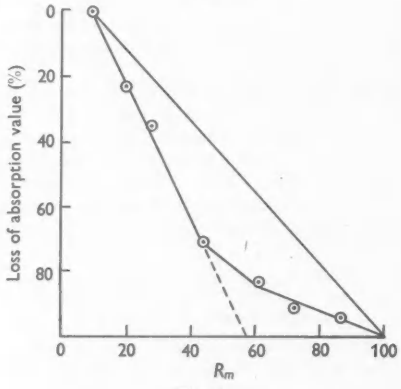

Fig. 3.

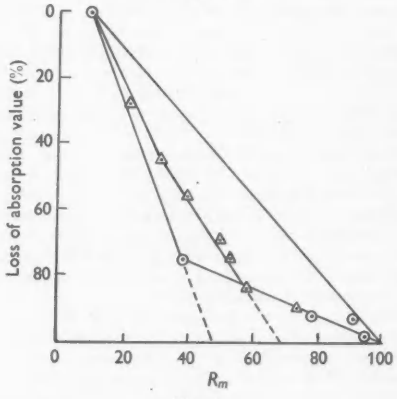

Fig. 4.

Figs. 1–4. Relationship between percentage loss of absorption value at 680 mμ. and increase of R_m during hydrolysis of amylose dextrins by crystalline β-amylase. Fig. 1. Dextrin P_1. Incubated at pH 3·6 and 21°. Time of half reaction, 2 min. 27 sec. Fig. 2. Dextrin P_2. Incubated at pH 3·6 and 21°. Time of half reaction, 21 min. Fig. 3. Dextrin R_1. Incubated at pH 6·8 and 32°. Time of half reaction, 9 min. 30 sec. Fig. 4. Dextrin R_2. Incubated with soya bean β-amylase. Fast reaction: pH 4·6, 32°. Time of half reaction, 14 min. (⊙); slow reaction: pH 5·8, 32°. Time of half reaction, 240 min. (△).

Figs. 5–7. Chromatograms showing progress of action of crystalline β-amylase on linear hexasaccharide at 21°. S = substrate. C = control mixture of maltose and maltotriose.

	R_m		
Chromatogram no.	Fig. 5	Fig. 6	Fig. 7
S	34	—	—
1	54	48	51
2	70	57	58
3	93	64	63
4	95	70	78
5	96	—	89
6	98	—	93
7	—	—	—
8	—	—	97
pH	3·6	3·6	5·0
Time of half reaction (min.)	2	10	10

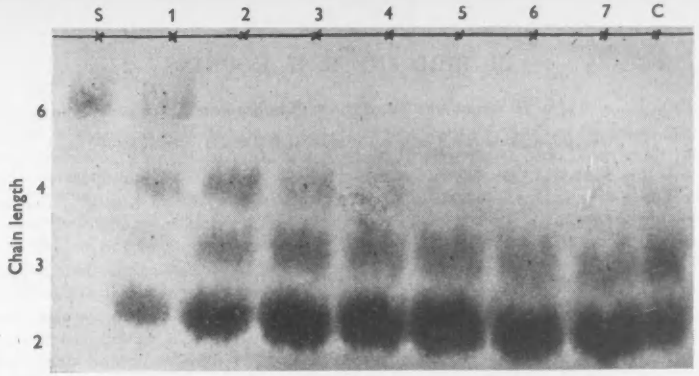

Fig. 5.

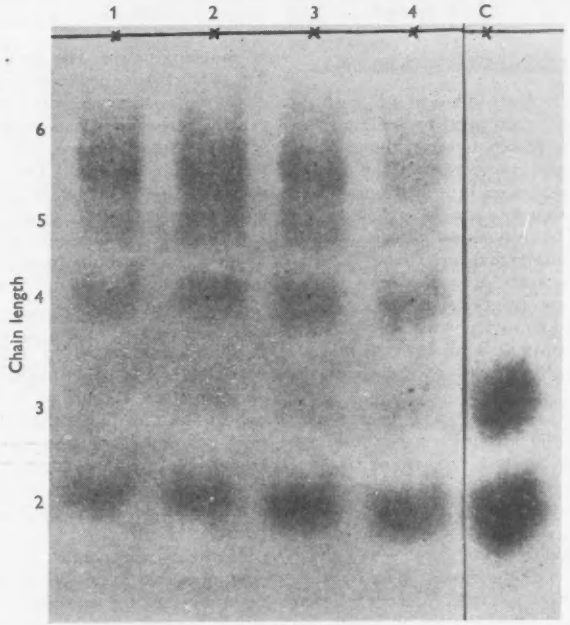

Fig. 6.

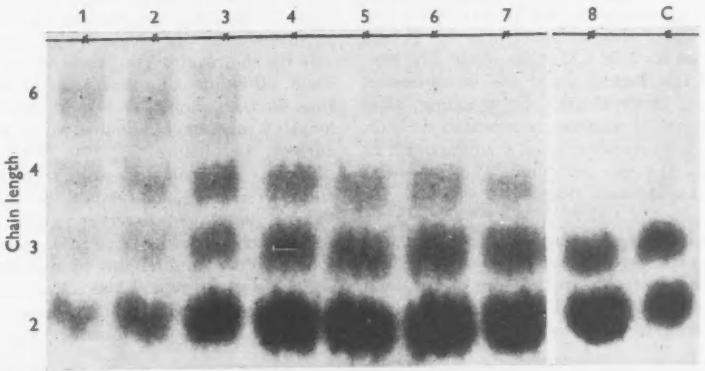

Fig. 7.

Figs. 5–7. For legends see foot of p. 142.

Table 1. *Fractionation of β-amylase/amylose hydrolysates*

(Absorption value (A.V.) ratio = A.V. read at 680 mμ./A.V. read at 490 mμ.)

Enzyme	pH		Maltose	Flocculum	Supernatant solution	Original amylose
Soya bean	5·8	Yield (%)	80	16·6	3·4	—
		A.V. ratio	—	—	1·62	—
Soya bean	4·6	Yield (%)	70·3	27·2	2·5	—
		A.V. ratio	—	—	1·2	3·0
		Chain length	—	260	—	480
Soya bean	4·6	Yield (%)	88	9·7	2·3	—
		A.V. ratio	—	—	0·76	—
		Chain length	—	—	20	—
Crystalline (sweet potato)	5·8	Yield (%)	62·5	34·4	3·1	—
		A.V. ratio	—	—	0·94	3·0
		Chain length	—	425	—	590

Fractionation of amylose undergoing β-amylolysis

Amylose (0·2%) was hydrolysed at 32° with β-amylase and the reaction stopped by boiling. The reaction mixture was frozen overnight and then allowed to thaw at room temperature; the flocculated sediment was separated by centrifuging and the supernatant solution was analysed. The conditions of the various hydrolyses and results of the fractionation are summarized in Table 1. Absorption values were read over the range 680–470 mμ., and the spectrograms indicated that short-chain material was under investigation. The ratio of the absorption values at 680 mμ. and at 490 mμ. is given in Table 1. For amylose this ratio is about 3·0.

When β-amylolysis of amylose was interrupted at stages earlier than R_m 50, no evidence was obtained of the presence in the reaction mixture of measurable quantities of short-chain fission products other than maltose. But at about R_m 60 and at subsequent stages, about 3% of products, the chain length of which ranged from 20 upwards as judged by their iodine stain, could be separated by freezing and thawing the reaction mixture. When a fraction of mean chain length 20–35 was obtained, its approximate chain length could be judged from the pattern of its absorption values by comparing this pattern with those of dextrins such as P_1, P_2 and others prepared for this and other work. The concentration of the dextrin could also be estimated by this means. Determination by weighing, after complete removal of maltose by repeated dialysis, was unsatisfactory since some red-staining dextrins slowly diffused through cellophan. The quantities stated in Table 1 were therefore calculated by difference.

For determination of chain length by periodate oxidation the supernatant fraction was exhaustively dialysed before analysis. In the case of the sediment, freedom from adherent maltose was ensured by reprecipitation until the supernatant liquid had no reducing power. The results showed that about 3% of the fission products were recovered as short-chain material as indicated by the iodine spectrogram. More could be extracted from the sediment at higher temperatures, but it was admixed with longer-chain material. The chain length of the residual amylose at 60–70% hydrolysis was much less than that of the original.

A control 'fractionation' of the original amylose yielded no carbohydrate in the supernatant fraction.

DISCUSSION

In considering the action of β-amylase on amylose-type dextrins of chain length 10 and upwards and giving iodine coloration, it is convenient to disregard the possibility that some of the molecules may contain barriers to the enzyme of the type responsible for the limited action on amylose reported by Peat, Pirt & Whelan (1952). Only a negligible portion of short-chain dextrins is protected in this way.

If the 'single chain' mechanism operates, the enzyme would hydrolyse a single chain completely before attacking another, and iodine coloration would diminish linearly with formation of maltose, finally disappearing at R_m 100. In Figs. 1–4 the extra diagonal line represents the theoretical progress under the 'single chain' mechanism.

With the 'multichain' mechanism, on the other hand, all iodine coloration would disappear by the time that all chains have been shortened to chain length 8 (and the blue value would disappear even earlier), i.e. before R_m 100. The most striking difference between the results to be expected from the two mechanisms should be obtained if the dextrin employed is composed of the shortest possible chains which give measurable colour with iodine. With 'multichain' hydrolysis, a dextrin of chain length 14 would lose all of its blue value on being shortened to 10 units, represented by a rise in R_m from 14·3 to 43. However, the production of

such a dextrin by fission of amylose entails difficulties in freeing it from dextrins of different chain length, a most important precaution. If, for instance, the product consisted of a mixture of dextrins of chain length 16 and 12, the removal of 2 units from all chains would be accompanied by fall in iodine colour values, as expected. Thereafter, the fission of dextrins of chain length 10 would contribute to reducing power without corresponding diminution of blue value. The graph, relating absorption value at 680 mμ. to R_m, would become less steep than hitherto. Some such effect is to be seen in Figs. 1–4. This source of error becomes more serious when relatively short-chain dextrins are used in this type of experiment. Furthermore, any contamination of the original material with achroic dextrin would result in the graph being from the outset less steep than it should have been, and the shorter the chain length of the dextrin employed, the more difficult is it to avoid such contamination. On the other hand, the greater the mean chain length, the nearer to 100 the value of R_m will be when the material becomes achroic. In fact dextrins of chain length 50 and upwards become almost indistinguishable from amylose in these experiments. Ordinary dialysis is not sufficiently selective if dextrins of chain length over 20 are to be purified. Bacterial α-amylase was used to hydrolyse the amylose, as, at the red-staining and subsequent stages, greater uniformity of chain length was expected than if acid was used. The methods adopted after some trials are given in the Experimental section.

The conclusion drawn from Figs. 1 to 4 is that the 'multichain' mechanism functions under the experimental conditions. The chain lengths of the dextrins ranged from over 30 in Fig. 1 to about 16 in Fig. 4, declining throughout the series. Corresponding to these, the initial linear parts of the graphs, extended to intersect the horizontal axis, did so at R_m 65, 65, 58 and 47, respectively. As expected, more decisive results were obtained with the dextrins R_1 and R_2 of shorter chain length. However, it also appears that the rate of the reaction exercised an influence. Fig. 4 shows results for a fast and a slow reaction with the same dextrin, R_2, the faster reaction giving the steeper curve. Another experiment, not reported in detail, in which soya bean β-amylase, which had been preheated in solution to 60° for 1 hr., gave results very similar to those of the slow reaction of Fig. 4. In these cases, however, it was noticed that the hydrolysis stopped at an unusually early stage. If the weak enzyme were for some reason to experience special difficulty in hydrolysing dextrins of chain length 6 or less, the 'single chain' mechanism could also account for the result since, in the case of either mechanism, the reaction would finish at about 75% R_m and the liquid would be achroic. This is the only way in which any of the results reported here can be reconciled with the 'single chain' hypothesis.

There are other distinctions between the manifestations of 'single' and 'multichain' mechanisms. With the former, the absorption value should fall linearly with increase in R_m, as already seen, and it should diminish at the same rate at all wavelengths. Table 2 shows that the blue values diminish more rapidly than the red and that both diminish much faster than would be expected on the 'single chain' hypothesis. On the 'single-chain' hypothesis also, one would expect the rate of maltose formation to fall during the reaction with diminishing molar concentration of substrate. However, it remained uniform (implying a zero-order reaction), scarcely diminishing by R_m 80. After this a marked retardation occurred. Allowing for iodine-staining dextrins still present, the residual achroic dextrins present at this stage would possess a mean chain length of 4.

The residual iodine-staining material at this stage includes some which cannot be hydrolysed by the pure β-enzyme. Indeed, it is of interest to note that a little red-staining residue (2–5%) was always present when β-amylolysis of these dextrins had reached its limit. The limit of such action on potato amylose is R_m 70 (Peat *et al.* 1952), and the obstruction to further action which occurs there evidently persists in some of the molecules of these iodine-staining dextrins. This was the case irrespective of whether the latter had been prepared by α-amylolytic or acid fission.

Table 2. *Loss in absorption values during β-amylolysis of dextrin* P_2

(The reaction mixture, containing dextrin P_2 (0·09%) and crystalline β-amylase, was incubated at pH 3·6 and 21°. The reaction was of zero order to R_m 50, and almost so to R_m 81·6. A slower reaction continued, reaching R_m 94 after 3 hr. Hydrolysis in column 2 is expressed as % of the range leading to R_m 81·6, i.e. 100% hydrolysis is attained at R_m 81·6.)

Time (min.)	Hydrolysis (%)	Loss in absorption value (%) at: (mμ.) 680	600	490	470
2	5·4	17	13	10	7
5	13·5	28	26	20	19
10	26·6	45	42	40	40
20	52·2	72	71	68	67
42	100	89	89	87	87

The chromatograms (Figs. 5–7), in which hexasaccharide was substrate, show the presence during hydrolysis of tetrasaccharide, the amount of which did not diminish until all signs of hexasaccharide had disappeared. If the 'multichain' mechanism functions, we should expect hexasaccharide concentration to decrease while tetrasaccharide and maltose concentrations increase. Meanwhile, R_m will rise from 33 to 66, but shortly before this hexasaccharide should have disappeared, since below a certain concentration, it would not show on the chromatogram. From R_m 66 onwards tetrasaccharide concentration should decrease while maltose concentration should continue to increase. The three chromatograms show these changes. The spots corresponding to maltotriose are due to the presence of relatively small quantities of this product derived from corresponding quantities of penta- and/or hepta-saccharide in the original substrates. The very rapid reaction (Fig. 5) shows the slow fission of maltotriose, which did not start to diminish until all tetrasaccharide had disappeared.

These results are in marked contrast to those of French *et al.* (1950) who worked with amyloheptaose. As it remained a possibility that the 'multichain' mechanism may have been due to the high rates of reaction, some reactions which were several times faster than others under otherwise similar conditions have been included in both sets of experiments; for example, the rate in Fig. 1 was 9 times as fast as in Fig. 2, while the rate in Fig. 5 was 5 times as fast as in Fig. 6. It was possible for the 'multichain' mechanism to function and yet, by increasing the quantity of enzyme without altering other conditions, to increase the rate several times.

Finally, the β-amylolysis of amylose itself, summarized in Table 1, indicates that quantities of short-chain fission products were formed during the reaction. Such would not pass a cellophan membrane in dialysis (except perhaps in the case of the supernatant fraction which contained 88 % of the theoretical yield of maltose) and may well be missed when sought by such experimental methods. Furthermore, such short-chain products would certainly be precipitated by *n*-butanol together with the longer-chain residue still present.

Under conditions which preclude all but the faintest activity of α-amylase or Z-enzyme, such as the use of crystalline β-amylase, the reaction becomes exceedingly slow at R_m 70 (Peat *et al.* 1952). The appearance of short-chain products at this stage is approached suggests that some chains are hydrolysed completely by the time that the reaction slows down, and that they are hydrolysed by the 'multichain' mechanism.

The presence of short-chain products in our reaction mixtures at 80 and 88 % hydrolysis by soya bean β-amylase could easily be attributed to the presence of traces of α-amylase. The Z-enzyme of Peat *et al.* (1952) could account for action proceeding beyond 70 % but not for short-chain fission products unless the 'multichain' mechanism is assumed. The hydrolysis with crystalline β-amylase to 62·5 %, still some way short of the arrest point with the crystalline enzyme, indicated some shortening of chain length (from 590 to 425) in the long-chain residue. It is not yet possible to state the nature of the barrier to action of the crystalline enzyme which occurs at 70–72 % with potato amylose (Peat *et al.* 1952) and is confirmed with our materials, but its bearing on the results of experiments such as this is evidently important.

SUMMARY

1. The hydrolysis of amylose dextrins of chain length 16–30 by soya bean and crystalline sweet-potato β-amylases has been followed by measurement of absorption value of iodine coloration and reducing power.

2. The relatively rapid disappearance of the iodine colour is consistent with a 'multichain' mechanism in which all chains are liable to be attacked.

3. Paper chromatographic analyses of the fission products of hexasaccharide by crystalline β-amylase indicate that the intermediate product, tetrasaccharide, accumulated until the hexasaccharide disappeared. Maltotriose was slowly hydrolysed.

4. Diminution in chain length of the residue and the presence of short-chain fission products during hydrolysis of amylose by β-amylase were observed and indicate a 'multichain' mechanism.

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The Formation of D-3-Phosphoglyceric Acid from Pentoses by *Escherichia coli*

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Whilst the breakdown of glucose to D-3-phosphoglyceric acid (PGA) has been observed in a number of micro-organisms (Werkman, Zoellner, Gilman & Reynolds, 1936; Stone & Werkman, 1936*a*, *b*, 1937; Wood, Stone & Werkman, 1937; Still, 1940), the formation of PGA from pentoses has so far only been observed in the case of *Propionibacterium pentosaceum* (Barker & Lipmann, 1949). The present investigation deals with the transformation of pentoses to PGA by *Escherichia coli*; it forms part of a study of the pathways of pentose fermentation. Since the fermentation of pentoses has been shown to be adaptive (Karström, 1938; Cohen & Scott, 1951; Cohen & Raff, 1951; Heald, 1952), the experiments were carried out with cells which had been grown upon media containing D-xylose and L-arabinose, respectively, and, for comparison, with cells which had been grown previously in the presence of glucose.

MATERIALS AND METHODS

Cultures. Cultures of *Esch. coli* (ATCC 9637) were maintained on 0·5% (w/v) Difco yeast extract slants with the addition of 0·2% (w/v) of the corresponding sugar. Cells from an 8-hr.-old agar slant culture were inoculated into 1·5 l. of a mineral medium (Cohen, 1949) contained in a 4 l. Erlenmeyer flask. Some experiments were carried out in a 0·5% Difco yeast extract liquid medium containing 0·05% sugar. The cultures were aerated vigorously and the cells harvested after 13–15 hr. and washed twice with 0·85% chilled KCl solution. Incubation temperature, 37°.

Materials. The following carbohydrates were used as substrates: D-glucose (C.P. analysed, Baker Chemical Co.); D-xylose, L-arabinose and D-lyxose (Pfanstiehl Chemical Co.); maltose, D-ribose and D-arabinose (Nutritional Biochemicals Corporation); glucose 1-phosphate (Bios Laboratories Inc.). Adenosine triphosphate (ATP) was used as the sodium salt, prepared from the commercial diharium salt (Delta Chemical Works).

Experimental conditions. The experimental conditions were similar to those described by Stone & Werkman (1937): wet cells, 0·35 or 0·70 g.; sugar, 100–500 μmoles; phosphate buffer (pH 6·8), 0·134 M; $MgCl_2$, $6H_2O$, 0·00143 M; acetaldehyde, 360 μmoles; NaF, 0·0086 M; toluene, 0·1 ml.; total volume, 3·5 ml. The mixture was shaken aerobically in a 25 ml. Erlenmeyer flask for 4 hr. at 37°. A 50% (w/v) trichloroacetic acid solution (0·4 ml.) was added and the precipitate removed by centrifuging. The supernatant was freed from inorganic phosphate by the addition of 0·4 ml. of magnesia mixture.

Analytical methods. Phosphoglyceric acid was determined polarimetrically (Meyerhof & Schultz, 1938), pentose by the orcinol reaction according to Drury (1948), and the total reducing sugar by the method of Folin & Malmros (1929).

RESULTS

Cells grown previously on pentose. The aerobic formation of PGA from pentose, glucose and maltose by cells which had been adapted to D-xylose and L-arabinose in mineral media is shown in Table 1. In all cases PGA was formed from maltose and glucose, whilst in the case of the pentoses, PGA was formed only from the sugar to which the cells were adapted; similar results were obtained with cells grown on yeast extract medium. Attempts to adapt the organism to grow in a synthetic medium containing D-ribose, by successive subculturing in the presence of this pentose, were unsuccessful.

Cells grown previously on glucose. Table 1 shows that cells grown in glucose-containing medium produced, under aerobic conditions, considerable amounts of PGA from glucose and maltose, whilst only small quantities were formed from L-arabinose, D-ribose, and D-lyxose. The results obtained with D-xylose and D-arabinose were surprising: not only was no PGA formed when small amounts of these sugars were used, but addition of larger amounts reduced the quantity of PGA formed to below that produced by the blank (PGA formed in absence of added substrate). Furthermore, in the case of L-arabinose-grown cells addition of D-arabinose and D-xylose decreased the endogenous PGA formation, whilst with xylose-grown cells only D-arabinose showed this effect. When the yeast extract medium was employed, the blank values were much higher, and consequently the effect was even more pronounced.

Table 1. *Aerobic formation of* D-3-*phosphoglyceric acid by cells of* Esch. coli *grown on* D-*glucose,* L-*arabinose and* D-*xylose*

(0·7 g. wet cells grown on mineral medium, buffer containing 0·134 M sodium and potassium phosphates, pH 6·8, 0·00143 M-$MgCl_2$, $6H_2O$, 0·0086 M-NaF, 360 μmoles acetaldehyde, 0·1 ml. toluene. Total volume, 3·5 ml. Incubation time, 4 hr. Temp. 37°.)

		Cells grown on D-glucose		Cells grown on L-arabinose		Cells grown on D-xylose	
Substrate	Concn. (μmoles)	PGA (μmoles)	ΔPGA* (μmoles)	PGA (μmoles)	ΔPGA (μmoles)	PGA (μmoles)	ΔPGA (μmoles)
Maltose	120	157·5	146·6	144·5	137·6	160·6	156·4
D-Glucose	120	83·3	72·4	79·0	72·1	80·0	76·8
D-Ribose	120	15·0	4·1	10·0	3·1	8·4	4·2
L-Arabinose	120	13·7	2·8	83·9	77·0	7·4	3·2
D-Arabinose	120	10·0	−0·9	9·5	2·6	3·0	−1·2
D-Arabinose	600	4·9	−6·0	0·7	−6·2	0·0	−4·2
D-Xylose	120	9·4	−1·5	6·9	0·0	35·2	31·0
D-Xylose	600	1·0	−9·9	0·0	−6·9	117·9	113·7
D-Lyxose	120	11·4	0·5	10·3	3·4	12·9	8·7
No addition	—	10·9	—	6·9	—	4·2	—

* ΔPGA = difference between PGA formed and control (incubated without sugar).

Table 2. *Formation of phosphoglyceric acid from glucose* 1-*phosphate in the presence or absence of* D-*xylose by glucose-grown cells of* Esch. coli

(0·18 g. wet cells grown on yeast extract medium; buffer containing 0·134 M sodium and potassium phosphates, pH 6·8, 0·00143 M-$MgCl_2$, $6H_2O$, 0·0086 M-NaF, 360 μmoles acetaldehyde, 0·1 ml. toluene. Total volume, 3·5 ml. Incubation time, 4 hr. Temp. 37°.)

Substrate	Concn. (μmoles)	PGA (μmoles)	ΔPGA* (μmoles)
None	—	2·6	—
D-Xylose	500	0·0	−2·6
Glucose 1-phosphate	7·8	8·5	—
Glucose 1-phosphate + D-xylose	7·8 500	0·0	−8·5
Glucose 1-phosphate	39	30·1	—
Glucose 1-phosphate + D-xylose	39 500	10·0	−20·1

* ΔPGA = difference between PGA formed and control (incubated without xylose).

Table 3. *Anaerobic formation of* D-3-*phosphoglyceric acid by* D-*xylose-grown cells of* Esch. coli

(0·35 g. wet cells grown on mineral medium, buffer containing 0·134 M sodium and potassium phosphates, pH 6·8, 0·00143 M-$MgCl_2$, $6H_2O$, 0·055 M-KF, 360 μmoles acetaldehyde, 25 μmoles ATP, 0·1 ml. toluene. Total volume, 3·5 ml. Incubation time, 4 hr. Temp. 37°.)

Substrate	Concn. (μmoles)	PGA (μmoles)	ΔPGA* (μmoles)
Maltose	120	89·0	88·5
D-Glucose	120	69·8	69·3
D-Ribose	120	2·9	2·4
L-Arabinose	120	3·9	3·4
D-Xylose	120	80·7	80·2
D-Xylose, no ATP	120	8·0	—
No sugar and ATP added	—	0·5	—

* ΔPGA = difference between PGA formed and control (incubated with ATP and without sugar).

Table 4. *Effect of phosphate and potassium ions on the formation of* D-3-*phosphoglyceric acid from xylose by xylose-grown cells of* Esch. coli

(0·35 g. wet cells grown on yeast extract medium, 0·00143 M-$MgCl_2$, $6H_2O$, 0·055 M-NaF, 360 μmoles acetaldehyde, 25 μmoles ATP, 0·1 ml. toluene. Total volume, 3·5 ml. Incubation time, 4 hr. Temp. 37°.)

Substrate	Concn. (μmoles)	Sodium and potassium phosphates (μmoles)	PGA (μmoles)
None	—	469	4·5
Xylose	120	117	34·6
Xylose	120	234	40·6
Xylose	120	469	62·3
Xylose	120	469*	81·9

* Sodium and potassium phosphates and NaF were replaced by 469 μmoles KH_2PO_4 and K_2HPO_4 and 0·055 M-KF.

It was thought that xylose might have been converted under these conditions into a phosphate ester. However, determination of the amount of pentose phosphate (orcinol reaction) in the barium-soluble, alcohol-insoluble fraction (Umbreit, Burris & Stauffer, 1945) showed that even less phosphorylated pentose was formed from xylose than in the blank. D-Xylose also inhibited the formation of PGA from glucose 1-phosphate (Table 2). In these experiments the quantity of cells used was decreased in order to reduce the formation of endogenous PGA.

Anaerobic PGA formation. The cells were grown in a mineral medium containing D-xylose as above, but the experiments were carried out under anaerobic conditions in evacuated Thunberg tubes. Table 3 shows that PGA was formed from xylose, glucose and maltose in the presence of ATP. In the case of xylose, 8 μmoles of PGA were formed without ATP as compared with 80 μmoles with ATP.

Table 5. *Pentose formation from glucose by glucose-grown cells of* Esch. coli

(0·5 g. wet cells grown on yeast extract medium, buffer containing 0·35 M sodium and potassium phosphates at pH 6·8, 0·00143 M-$MgCl_2$, $6H_2O$, 0·0086 M-NaF, 300 μmoles sodium fumarate, 0·1 ml. toluene. Total volume, 3·5 ml. Incubation time, 4 hr. Temp. 37°.)

Substrate	PGA (μmoles)	Pentose (μmoles) 0 hr.	4 hr.	Pentose formed	Δ Pentose*	Pentose phosphate (μmoles)	Glucose utilized (μmoles)
None	24·1	1·5	10·8	9·3	—	2·1	—
Glucose, 160 μmoles	37·6	1·5	19·4	17·9	8·6	2·7	21·2

* Δ Pentose = difference between pentose formed from glucose and in the control (incubated without glucose).

Table 6. *Determination of free and protein-bound pentose in glucose-grown cells of* Esch. coli

(Bacterial cell suspension as in Table 5.)

Substrate	(1) Free pentose (μmoles) (0 hr.)	(4 hr.)	(2) Change in free pentose (μmoles)	(3) Protein-bound pentose* (μmoles) (0 hr.)	(4 hr.)	(4) Change in protein-bound pentose (μmoles)	(5) Increase of pentose formation (μmoles) ((2) − (4))
None	1·5	18·0	16·5	12·0	0·0	−12·0	4·5
Glucose, 120 μmoles	1·4	24·2	22·8	12·2	0·3	−11·9	10·9

* Protein-bound pentose = total pentose − free pentose.

Effect of potassium and phosphate. In the anaerobic experiments with xylose as substrate, replacement of the sodium ion of the buffer (Na_2HPO_4) by potassium (K_2HPO_4) increased PGA formation by 28 %. A relationship was found to exist between the increase of the phosphate concentration and the production of PGA (Table 4).

Isolation of PGA. From experiments with D-xylose, D-3-phosphoglyceric acid was isolated in the form of its acid barium salt, $[\alpha]_D^{20} - 754°$ in the presence of molybdate (Meyerhof & Schultz (1938) give $[\alpha]_D^{20} - 745°$). (Found: P, 8·5. $C_3H_5O_7PBa$, $2H_2O$ requires 8·7 %.)

Formation of pentose from glucose. The formation of PGA from glucose by glucose-grown cells of *Esch. coli* is not the only process occurring. In addition to PGA, small amounts (0·1–0·4 μmoles) of pentose were formed when glucose-grown cells were incubated in the presence of glucose with fumarate as the oxidizing agent. Part of this pentose was found in the barium-soluble, alcohol-insoluble fraction (Umbreit *et al.* 1945) and probably occurs as a phosphate ester. The results of a typical experiment are given in Table 5.

The possibility that the net formation of pentose (Δ pentose in Table 5) was due to breakdown of the nucleic acids of the cell, was eliminated by an experiment using the method of Schneider (1945). Samples of the suspensions (0·1 ml.) with and without glucose, as described in Table 5, were removed before the incubation and 4 hr. later. To each sample, 4·9 ml. of a 5 % (w/v) trichloroacetic acid solution was added. The acid-soluble pentose was determined in the filtrate after treatment with trichloroacetic acid at 5° for 30 min., and the total pentose (acid-soluble plus nucleic acid pentose) was determined after the treatment at 90° for 15 min.

The data given in Table 6 show that the net increase of 6·4 μM of the acid-soluble pentose formed in the presence of glucose (increase of pentose formed in the presence of glucose minus increase of pentose formed in its absence) (column 5) did not derive from the nucleic acids of the bacterial cells.

DISCUSSION

In the experiments described here, PGA is formed from pentoses, although not to the extent of 1 mole per mole of pentose utilized; the maximum yield obtained was 80 % of that quantity. The difference may be attributed to the incompleteness of the inhibition caused by fluoride, rather than to a mechanism of pentose degradation different from the fission into C_2 and C_3.

Stone & Werkman (1936*a*, *b*, 1937) have shown that toluene-treated cells of *Esch. coli* form substantial amounts of PGA from glucose in the presence of hexose diphosphate (HDP) under both aerobic and anaerobic conditions. In the present investigation, it was found that the anaerobic breakdown of xylose by toluene-treated suspensions of *Esch. coli* requires the addition of small quantities of ATP, whilst in the case of the aerobic breakdown no additional ATP is required. It is assumed that the aerobic system can generate energy-rich phosphate bonds and thus dispense with an extraneous source of them.

In its ability to transform pentoses into PGA, *Esch. coli* resembles *Propionibacterium pentosaceum* (Barker & Lipmann, 1949). The enzyme system involved in this process (or at least one enzyme of this system) is adaptive in nature, as the conversion of a pentose to PGA was only observed in cells which had been grown previously in presence of that pentose (see Ley & Cornut, 1951; Heald, 1952).

In striking contrast to this result stands the observation that glucose-grown cells of *Esch. coli* do not convert D-xylose and D-arabinose into PGA (L-arabinose and D-ribose give small amounts of the acid); moreover, when D-xylose and D-arabinose are used in large enough amounts, the quantity of PGA formed is less than that which the cells produce in absence of any added substrate. L-Arabinose-grown cells behave similarly; but in the case of D-xylose-grown cells, only D-arabinose inhibits the production of PGA. It has been shown that this effect is not due to a phosphorylation of the inhibitory pentose. The endogenous PGA is presumably formed from the reserve carbohydrate of the bacterial cells by glycolysis. The fact that xylose inhibits the formation of PGA from glucose 1-phosphate tends to indicate that at least one of the effects of the xylose consists in interference in a reaction subsequent to the phosphorolysis of the cell carbohydrate by phosphorylase.

This inhibitory action of pentose recalls the finding of Doudoroff, Hassid, Putman, Potter & Lederberg (1949) that the amylomaltase of *Esch. coli* (Monod & Torriani, 1948) is inhibited by D-xylose. However, whilst amylomaltase is not affected by D-arabinose, in the present investigation this sugar behaved like D-xylose. Thus, the resemblance between the two phenomena is incomplete, and no definite conclusion can be drawn as to the point at which the pentose molecule blocks the pathway of the formation of endogenous PGA.

In the experiments with glucose, it was found that *Esch. coli* transforms this sugar not only into PGA, but also into significant amounts (10–40 μmoles/100 μmoles of glucose utilized) of pentose, partly in the form of its phosphate. This fact is in accord with the observation of Cohen (1951) and Scott & Cohen (1951) that glucose can be oxidized via glucose 6-phosphate and 6-phosphogluconic acid to a pentose phosphate and carbon dioxide. It is likely that the amount of pentose found does not represent the total quantity formed from glucose, as part of the pentose will be transformed into PGA under the present experimental conditions.

SUMMARY

1. *Escherichia coli*, adapted to D-xylose or L-arabinose, produces under aerobic conditions D-3-phosphoglyceric acid (PGA) from maltose, D-glucose and from the pentose to which it had been adapted.

2. *Esch. coli*, grown previously in the presence of glucose, gives significant amounts of PGA when incubated aerobically with D-glucose and maltose, and very small amounts when incubated with L-arabinose, D-ribose, D-xylose, or D-lyxose.

3. D-Xylose and D-arabinose decrease the quantity of endogenous PGA formed by cells grown on D-glucose and L-arabinose; for D-xylose-grown cells, only D-arabinose shows a similar effect.

4. A large amount of PGA is formed from xylose by xylose-grown cells under anaerobic conditions only when adenosine triphosphate is added.

5. In the presence of potassium ions, more PGA is formed than in the presence of sodium ions.

6. *Esch. coli* transforms glucose partly into a pentose and pentose phosphate.

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The Utilization of Non-protein Nitrogen in the Bovine Rumen

8. THE NUTRITIVE VALUE OF THE PROTEINS OF PREPARATIONS OF DRIED RUMEN BACTERIA, RUMEN PROTOZOA AND BREWER'S YEAST FOR RATS

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Earlier papers (cf. Pearson & Smith, 1943) in this series described the considerable protein synthesis which occurs when rumen liquid, obtained from a bullock with a permanent fistula, is incubated *in vitro* with non-protein nitrogen and readily available carbohydrate. Smith & Baker (1944) later correlated this protein synthesis with increase in numbers of iodophilic bacteria. Subsequent work showed that the protein of a dried preparation of rumen bacteria, approximately 40% of which had been synthesized during incubation *in vitro*, had, when tested in rats, the high biological value of 88 (McNaught, Smith, Henry & Kon, 1950). Its true digestibility was, however, only 73%. In reviewing the subject of nitrogen metabolism in the rumen, McNaught & Smith (1947) pointed out that, although the power of the rumen protozoa to synthesize protein had not been specifically demonstrated (Schmid, 1939; Smith & Baker, 1944), it was possible that they served a useful purpose by assimilating bacterial protein and thus rendering it more available to the host. The observation of Usuelli & Fiorini (1938), that growth of chicks was progressively improved by additions to their diet of bacteria and protozoa from the rumen of sheep, is in accord with the hypothesis that rumen protozoa contribute to the nutrition of the host animal. Johnson, Hamilton, Robinson & Garey (1944) found that the protein of rumen protozoa was better digested by the rat than that of rumen bacteria. They did not attach much importance to this finding because of the doubtful purity of their bacterial preparation. They had so little material that they used too few rats to make a satisfactory estimate of the relative nutritive values of rumen protozoa and bacteria. Since a procedure for isolating protozoa in adequate amounts from rumen contents has now been devised, it seemed desirable to repeat the earlier tests on a rumen-bacterial preparation, simultaneously with tests on the protozoa. A third source of microbial protein, dried brewer's yeast, was tested at the same time for comparison. The present communication describes the preparation of the dried protozoa at the Hannah Institute and the biological tests carried out at the National Institute for Research in Dairying.

EXPERIMENTAL

Preparation of bacteria

This was as described by McNaught *et al.* (1950). During the collection period a cow and a bullock with rumen fistulas were maintained on daily rations consisting of 6 lb. of a concentrate mixture (equal parts of dried grass, oats and beans) and 10 lb. hay. Glucose (1%) instead of maltose was added to the rumen liquid before incubation. An electrical homogenizer was used to suspend the bacteria in ethanol. In the present preparation 118 l. of rumen liquid yielded 283 g. of dry bacteria, an average yield of 2·4 g./l. This preparation was mixed with 53 g. from the previous preparation which had been stored at 0–4° for 5–6 yr.

Preparation of protozoa

Since it is well known that the number of protozoa in the rumen can to a large extent be controlled by the diet of the animal and in particular by the level of dietary protein (Ferber, 1929; Mangold, 1929; Louw & van der Wath, 1943; Williams & Moir, 1951), it was essential to establish and maintain a dense population by a suitable diet. The diet that had been fed to the fistulated animals during the preparation of the rumen bacteria (McNaught *et al.* 1950) was found to be satisfactory. The method of preparation recommended by Johnson *et al.* (1944) did not prove satisfactory in our hands, as we were unable adequately to separate fibre from the protozoa.

The most efficient separation was achieved by allowing the liquid, obtained by squeezing rumen contents through muslin, to stand in cylinders in a deep water bath, thermostatically controlled at 39°. Within a short time (about 15 min.) most of the dark-coloured fibrous material rose to the surface and formed a compact layer. The protozoa formed a whitish layer at the bottom of the cylinder and could be separated by decantation. Preliminary experiments showed that addition of some soluble carbohydrate to the liquid accelerated the separation and tended to give a larger volume of protozoa. Dilution of the rumen liquid also tended to hasten separation. Oxford (1951) has shown that rumen protozoa very rapidly ferment certain sugars

Table 1. *Yields of protozoa from samples of rumen liquid withdrawn from animals at different times throughout the day*

(For experimental details see text.)

Time	Protozoa isolated without added sugar: Dry matter (mg./l.)	N as % dry matter	Yield of N (mg./l.)	Protozoa isolated in presence of added sugar: Dry matter (mg./l.)	N as % dry matter	Yield of N (mg./l.)
Animal I						
7.30 a.m.*	117	8·7	10·2	193	5·5	10·6
9.30 a.m.†	116	8·9	10·3	471	4·5	21·2
10.30 a.m.	231	8·7	20·1	506	3·7	18·7
12.00 midday	324	6·9	22·3	370	4·2	15·5
2.45 p.m.	163	6·2	10·1	248	4·2	10·4
5.00 p.m.	104	6·5	6·7	317	4·2	13·3
Animal II						
7.30 a.m.*	189	9·8	18·4	615	4·2	25·5
9.30 a.m.†	259	9·5	24·4	799	4·7	37·2
10.30 a.m.	478	7·6	36·5	747	4·2	31·5
12.00 midday	300	6·8	20·5	422	3·8	15·9
2.45 p.m.	420	7·0	29·4	436	4·3	18·5
5.00 p.m.	278	7·2	20·0	450	5·3	23·6

* Hay fed after removal of sample.
† Concentrates (3 lb.) given after removal of sample.

and store a glucosan (Masson & Oxford, 1951; Sugden & Oxford, 1952). It was therefore of interest to determine whether more bulky yields of protozoa obtained by addition of glucose did, in fact, contain more protein than protozoa isolated in the absence of glucose. Furthermore, the ability of rumen liquid to ferment sugar varies considerably throughout the day in relation to time of feeding (Oxford, 1951). Consequently, it was important to know whether yields of protozoa obtained by our technique varied in a similar manner. In a preliminary experiment, samples of rumen contents were withdrawn at the times shown in Table 1. After filtration through muslin, the liquid was divided into two portions. One portion was diluted with one-third of its volume of warm water (approx. 39°) and the other with the same volume of 0·5% (w/v) glucose. The liquids were then poured into cylinders and allowed to stand in the bath. When the protozoa had ceased streaming out of the floating, fibrous layer, the liquid was carefully decanted and the well-defined protozoal layer was transferred to a beaker. The liquid was well mixed and allowed to settle twice more to take out any protozoa trapped in the fibrous material during the first settling. The combined protozoal fractions were suspended in cold tap water and allowed to settle in the beaker. This procedure was repeated until the supernatant layer was quite colourless. The subsequent procedure was as for the bacteria, i.e. the protozoa were twice suspended in ethanol and twice in ether. The total yields of protozoa and of protein from the two animals are shown in Table 1.

It is clear that maximum yields are obtained 2–4 hr. after the 7.30 a.m. feed. The addition of sugar to samples obtained during this period resulted in a considerable increase in the total yield of protozoa but had a negligible effect on that of nitrogen. Analyses showed a substantial storage of polysaccharide in the protozoa which had been allowed to settle in the presence of added glucose and, although an increase in the total protozoal nitrogen was not consistently obtained, glucose was subsequently added to all preparations to accelerate the settling of the protozoa. By this technique up to 24 l. of rumen contents, obtained from the two animals 2–4 hr. after feeding, could be processed each day. In all, 878 l. were treated in this way giving a total yield of 447 g. of protozoa, an average yield of 0·5 g./l.

Table 2. *Percentage composition of dried preparations of rumen bacteria and protozoa*

Component	Rumen bacteria	Rumen protozoa
Moisture	11·2	8·2
Protein (N × 6·25)	41·8	26·5
Polysaccharide*	32·0	62·1
Ash	6·7	2·3
Lipid	2·4	1·4
Crude fibre	2·2	1·7
Total	96·3	102·2

* As starch, corrected for pentose.

Dried brewer's yeast

This was obtained from Arthur Guinness Son and Co. (Dublin) Ltd., Park Royal, London, N.W. 10.

Analyses of the preparations

The preparations were analysed for moisture, nitrogen, polysaccharide, ash, lipid and crude fibre. Moisture was determined by the oven method, nitrogen by the Kjeldahl procedure and polysaccharide by the method recommended by the Association of Official Agricultural Chemists (1945) for starch, in which reducing power is estimated after acid hydrolysis. Pentose was also measured and correction made for it in calculation of the polysaccharides. Lipid material was estimated by saponification followed by extraction, ash by ignition and crude fibre by the method of Whitehouse, Zarow & Shay (1945). The results are recorded in Table 2, where it is shown that they account for 96% of the mass of the bacterial preparation. The corresponding figure for the protozoal preparation was 102%, the extra 2% probably being due to an overestimation of polysaccharide.

Table 3. *Individual nitrogen-metabolism data on twelve rats for 6-day periods showing the biological values and true digestibilities of the proteins* (N × 6·25) *of rumen bacteria, rumen protozoa and dried brewer's yeast*

Rat no.*	Protein tested†	Wt. of rat (g.) Initial	Wt. of rat (g.) Final	Diet intake (g.)	N‡ intake (mg.)	Faecal N (mg.)	Urinary N (mg.)	Biological value	True digestibility
				Initial low egg-nitrogen period					
1	—	54	59	45·71	306·3	65·2	58·3	—	—
2	—	53	54	36·47	244·3	76·5	50·6	—	—
3	—	53	61	43·53	291·7	70·0	54·7	—	—
4	—	55	55	30·16	202·1	76·1	52·9	—	—
5	—	55	62	47·29	316·8	84·2	50·1	—	—
6	—	57	64	44·15	295·8	82·6	62·7	—	—
7	—	63	70	46·48	311·4	88·6	54·6	—	—
8	—	61	61	33·64	225·4	68·4	48·1	—	—
9	—	56	64	48·14	322·5	92·6	64·0	—	—
10	—	54	59	44·62	299·0	69·6	60·3	—	—
11	—	49	55	37·77	253·1	60·7	63·9	—	—
12	—	53	61	47·84	320·5	72·1	59·9	—	—
				1st Experimental period					
1	B	64	70	38·97	483·5	200·1	107·2	87·6	71·5
2	P	62	69	36·47	417·9	129·0	116·6	83·6	87·4
3	Y	68	68	31·33	395·0	121·3	137·4	77·5	83·3
4	B	62	66	30·67	380·6	153·2	95·1	88·2	80·0
5	P	70	84	47·48	544·2	125·8	118·4	89·0	92·2
6	Y	73	75	39·32	495·8	155·9	161·7	78·0	83·0
7	B	78	78	33·33	413·5	167·0	108·1	85·4	74·9
8	P	70	84	47·96	549·5	116·2	110·0	90·2	94·5
9	Y	71	71	29·83	376·1	123·8	128·1	81·5	82·8
10	B	66	71	43·49	539·6	187·3	136·1	83·5	78·6
11	P	63	77	48·98	561·3	147·9	163·6	82·9	88·7
12	Y	71	80	48·81	615·5	186·0	188·0	77·9	82·4
				2nd Experimental period					
2	Y	71	79	45·00	567·4	182·0	221·4	66·0	84·4
3	B	70	74	44·50	552·2	229·3	142·1	81·1	73·9
1	P	76	86	49·50	567·2	163·7	191·5	74·0	80·9
5	Y	90	96	57·00	718·8	201·6	278·4	66·7	85·8
6	B	78	86	48·00	595·5	211·7	153·2	82·9	78·7
4	P	72	88	51·00	584·5	140·3	144·7	87·2	97·8
8	Y	88	94	57·00	718·8	217·4	286·6	63·0	82·1
9	B	71	77	47·50	589·3	285·4	177·4	72·7	68·0
7	P	80	93	51·00	584·5	134·7	192·8	76·6	93·5
11	Y	81	91	56·85	716·8	201·4	292·2	68·5	86·4
12	B	82	88	54·50	676·3	277·8	192·9	77·0	72·4
10	P	79	89	55·50	636·0	147·8	209·5	76·6	91·9
				3rd Experimental period					
3	P	83	93	49·99	572·9	174·9	196·4	76·3	87·4
1	Y	93	100	61·98	781·6	266·4	301·2	65·3	81·3
2	B	87	93	54·49	676·1	313·0	165·6	78·6	70·4
6	P	95	108	59·97	687·3	152·5	211·2	80·0	92·8
4	Y	95	102	61·93	780·9	255·1	318·7	65·6	87·1
5	B	103	115	67·99	843·7	365·0	189·5	82·0	70·6
9	P	85	99	59·98	687·4	192·8	206·7	79·2	90·3
7	Y	101	112	62·98	794·3	240·1	146·1	90·7	84·7
8	B	103	117	66·95	830·7	343·8	222·2	75·4	69·2
12	P	99	115	65·97	756·1	165·8	341·5	65·0	93·3
10	Y	99	107	63·99	806·9	275·2	336·4	60·3	80·4
11	B	98	108	63·48	787·5	333·4	220·9	77·8	73·3
				Final low egg-nitrogen period					
1	—	107	113	61·75	413·7	129·3	84·5	—	—
2	—	99	105	53·67	359·6	111·5	64·0	—	—
3	—	98	102	47·25	316·6	103·8	85·1	—	—
4	—	107	107	44·24	296·4	110·3	87·9	—	—
5	—	123	137	77·47	519·0	131·0	94·4	—	—
6	—	116	130	73·48	492·3	123·4	92·6	—	—
7	—	122	136	75·98	509·1	143·4	99·4	—	—
8	—	123	137	75·47	505·6	81·6	93·1	—	—
9	—	108	120	71·33	477·9	152·9	88·1	—	—
10	—	117	131	72·65	486·8	137·8	82·8	—	—
11	—	115	129	73·45	492·1	151·2	96·5	—	—
12	—	124	138	77·46	519·0	140·9	109·0	—	—

* Rat nos. 1, 2, 3 = litter 1; 4, 5, 6 = litter 2; 7, 8, 9 = litter 3; 10, 11, 12 = litter 4.

† B = rumen bacteria; P = rumen protozoa; Y = dried brewer's yeast.

‡ Nitrogen content of diets: low egg-nitrogen, 0·67%; rumen bacteria, 1·24%; rumen protozoa, 1·15%; dried brewer's yeast, 1·26%.

Table 4. *Mean results for groups of twelve rats for the biological values, true digestibilities and net utilization values of the proteins* (N × 6·25) *of rumen bacteria, rumen protozoa and dried brewer's yeast*

Protein	Biological value	True digestibility	Net utilization (biological value × true digestibility/100)
Dried rumen bacteria	81	74	60
Dried rumen protozoa	80	91	73
Dried brewer's yeast	72	84	60
Pooled S.E.	±1·53	±0·95	—

Purity of the preparations

The fibre content of the earlier bacterial preparation, 0·37 %, was taken as an indication of contamination with vegetable material (McNaught *et al.* 1950). The higher value of 2·2 % in the present sample might indicate greater contamination. It should be pointed out, however, that the present value was obtained by the method of Whitehouse *et al.* (1945), which is simpler than the double-digestion method of the Association of Official Agricultural Chemists (1945) used previously. A sample of the first preparation when analysed by the method of Whitehouse *et al.* (1945) gave a value of 2·06 % as compared with 0·37 % by the other method. This rather suggests that it is not true fibre that is being estimated, for the two methods agree well when used to determine fibre in feeding stuffs (Owen, 1946). When examined microscopically, both preparations appeared to contain only traces of fibre.

Microscopic examination of the protozoal preparation showed that it contained a considerable amount of extracellular material. At first this was thought to be contaminating bacteria, but further investigation showed that the material was probably the 'glucosan' granules from ruptured holotrich ciliates reported by Oxford (1951).

Biological tests

The biological value and true digestibility of the proteins of the rumen preparations and of the dried yeast were determined in litter-mate comparisons on four litters of three weanling female rats, at an 8 % level of protein intake, by the method of Mitchell (Mitchell, 1924*a*; Mitchell & Carman, 1926) as used in this laboratory (Henry, Kon & Watson, 1937; Henry, Kon, Lea & White, 1948). In each litter the rats received each diet in turn according to a Latin-square design.

The basal, nitrogen-free diet contained: cane sugar 12, margarine fat 10, potato starch 10, rice starch 64 and salt mixture (de Loureiro, 1931) 4 %. (This salt mixture is the same as that described by Campbell, Olley & Blewett (1949) except that it contains no $ZnSO_4$.) To bring the protein content of this diet to 8 % the following percentages of the test substances were added to it at the expense of rice starch: rumen bacteria, 19·0; rumen protozoa, 31·2; brewer's yeast, 20·6.

The results of the test are shown in Tables 3 and 4.

DISCUSSION

The estimates of the biological value and true digestibility of the bacterial protein agree satisfactorily with those previously obtained by us for a different sample of rumen bacteria (McNaught *et al.* 1950). The biological value of 81 now obtained is not essentially different from the earlier one of 88; we have noted similar variations in tests done at different times on the same sample of dried skim milk (cf. Henry, Kosterlitz & Quenouille, 1953). The present value is closely similar to those of 78 and 79 reported for two samples of bacteria isolated from the rumen of sheep by Reed, Moir & Underwood (1949) in Western Australia. The true digestibilities of 74 and 73, respectively, found now and in our previous test, are higher than those reported by the Australian workers (65 and 62). Although the biological value of the protozoal protein (80) does not differ from that of the bacteria, the true digestibility of 90 is markedly higher. The biological value of 72 found for the yeast is lower than the values for the bacteria and protozoa but falls within the range of 45–86 reported for yeasts in the literature (Goyco & Asenjo, 1947, 1949; Hughes & Hauge, 1945; Mitchell, 1924*b*; Sure & House, 1949). The true digestibility of 84 for the yeast is intermediate between the figures for bacteria and protozoa; it falls well within the range of 80–90 reported for yeasts by the above workers. It is of interest that the net utilization (biological value × true digestibility/100) for both the bacteria and the yeast is 60 %. The protozoa, with their higher digestibility, have a value of 73 %. Thus, in these tests, protozoa were superior to both rumen bacteria and yeast.

For many years the role of the protozoa in ruminant metabolism has been under investigation, and one of us (Owen, 1947*a*, *b*, 1951, 1954), in reviewing the status of rumen micro-organisms, has summarized the more recent work on the assessment of the value of protozoa. Becker (1929) and Becker & Everett (1930) observed that sheep defaunated with copper sulphate thrived better than untreated controls, suggesting that the protozoa were of no value to the host. Copper sulphate, however, not only would remove the protozoa in the rumen but would also increase the animal's thrift by acting as a vermifuge. There is, in addition, the possibility that Becker's control animals were not receiving optimum amounts of copper, the essential nature of which element was not then fully appreciated. Van der Wath & Myburgh (1941), using the

same defaunation technique, could not demonstrate an essential role for the protozoa in cellulose digestion, although it is known from the work of Hungate (1942, 1943) that at least one rumen protozoan (*Diplodinium* sp.) can digest cellulose. More recently, Sugden & Oxford (1952) found that the holotrich ciliates of the sheep's rumen could not use soluble starch for growth, though they could use inulin or a bacterial levan. It is therefore doubtful to what extent the protozoa play a part in the carbohydrate metabolism of the rumen. Their part in nitrogen metabolism also is rather obscure. The present investigation has indeed shown that the protozoa provide the host with a readily digestible protein of high biological value. Nevertheless, the final assessment of the benefit, if any, of its alimentary protozoa to the host can be made only after it is known from what nitrogen source the protozoa elaborate protein, and at what rate this protein becomes available through autolysis and digestion. Whether rumen protozoa can use non-protein nitrogen for protein synthesis is a matter for further study. They may depend entirely on the protein of the host's fodder or of the rumen bacteria, or their growth requirements may be satisfied by a less complex nitrogen source, such as amino acids, or even inorganic nitrogen in the presence of an organic carbon source (Hall, 1941). Whereas the biological values of the bacteria and protozoa have been shown to be the same, lower values of 60–70 have been reported for the usual feeding stuffs such as grass, oats, etc. (Block & Mitchell, 1946; Bartlett *et al.* 1938). Utilization of such food protein would involve some amino acid synthesis to yield protein of the higher biological value found in the present experiments. Thus the alimentary protozoa of the ruminant may well enhance the value of food or of bacterial protein by increasing its digestibility.

Though the yield of protozoa in the present experiments (0·5 g./l. rumen contents) appears to be small, the method of isolation was by no means quantitative nor did it separate the smaller protozoa. Estimates of the amount of protozoal material in the rumen have been discussed by Mangold (1929), who quotes Ferber (1929) as having found 4·4–8·7 % of protozoa in the dry matter of rumen contents. He also quotes Schwarz (1925) as estimating that 20–30 % of the nitrogen in the rumen contents of slaughterhouse cattle was due to protozoa. This estimate was made by assuming that all the nitrogen brought into solution by pouring a pepsin-hydrochloric acid mixture through a filter paper containing rumen contents was protozoal. Masson & Oxford (1951) could find only 1 % of protozoal starch ('glucosan') in a sheep's whole rumen contents but this estimate, like those of Ferber (1929) and of Schwarz (1925), gives no measure of the rate at which the total protozoal products are passing on to the abomasum or are being autolysed in the rumen itself. If Schwarz's results are correct or even if the lesser ones of Ferber are a better estimate, then the difference of utilization between protozoal and bacterial protein may well be of importance to the host.

SUMMARY

1. A sample of dried bacteria (283 g.) was prepared from bovine rumen liquid, by the method of McNaught *et al.* (1950). A sample of dried protozoa (447 g.) was prepared from rumen contents by straining them through muslin, diluting the strained liquid with glucose solution and allowing this liquid to settle at 39°. The protozoal layer was separated by decantation. These preparations, when dried, contained 41·8 % and 26·5 % of crude protein, respectively.

2. The biological value and true digestibility of the bacterial and protozoal protein were determined at an 8 % level of protein intake on groups of twelve young female rats by the balance-sheet method. Dried brewer's yeast was also included in the trials. Biological values of 81, 80 and 72 were obtained for the proteins of bacteria, protozoa and yeast, respectively. The corresponding values for true digestibility were 74, 91 and 84 %.

3. These results suggest that the conversion of bacterial or dietary protein into protozoal protein in the rumen is advantageous to the host animal.

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Studies on the Biosynthesis of Blood Pigments

1. HAEM SYNTHESIS IN HAEMOLYSED ERYTHROCYTES OF CHICKEN BLOOD

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In recent years, the isotope tracer method has been used extensively to study the biosynthesis of haem. This has shown that glycine nitrogen probably supplies all four haem nitrogens (Wittenberg & Shemin, 1949; Muir & Neuberger, 1949), and that glycine-α-carbon is used for the four methene bridges and for one carbon atom in each pyrrole nucleus (Muir & Neuberger, 1950; Wittenberg & Shemin, 1950). The remainder of the carbon atoms can be furnished by acetate (Shemin & Wittenberg, 1951). For these studies either whole animals or intact avian erythrocytes were used. For detailed studies of the mechanism, a system without cell-permeability barriers would be of obvious advantage. The synthesis can be studied in rabbit bone marrow 'homogenates' (Altman, Salomon & Noonan, 1949) and in rabbit-spleen 'homogenates' (Altman & Salomon, 1950). It appeared to us that, if active preparations could be obtained, haemolysed avian erythrocytes should provide a particularly convenient system.

In the present work, conditions are described for the preparation, from the blood of domestic fowls, of completely haemolysed erythrocytes which retain a high proportion of the synthetic activity of the intact cells, as measured by the use of [α-^{14}C]-glycine. The rate of haem synthesis has been studied under various conditions, including the presence of some haemopoietic factors. It has also been possible to show a net increase of free porphyrin on incubation. A preliminary report of this work has been made (Falk & Dresel, 1952). Haem synthesis has been obtained in similar preparations by London & Yamasaki (1952).

MATERIALS

The blood used was taken from normal adult domestic fowls (*Gallus domesticus*) pooled for marketing. Approximately 500 ml. blood was collected into 2 ml. 0·9% NaCl, containing 15 mg. penicillin G, 15 mg. streptomycin and 20 mg. heparin, and was used the same day. In one experiment, blood from vitamin B_{12}-deficient, 4-week-old chicks, and from the corresponding controls, was used. The parent hens had been on a diet containing all-vegetable protein for about 18 months. The 'B_{12}-deficient' chicks were reared on a B_{12}-free diet (Coates, Harrison & Kon, 1949), whereas the

controls were fed 4 μg. vitamin B_{12}/100 g. food throughout their lives. The mean weights of the ten B_{12}-deficient and ten B_{12}-supplemented chicks were 164 and 262 g., respectively. We are grateful to Dr M. Coates (National Institute for Research in Dairying, Reading) for this material.

Boiled yeast extract. Moist baker's yeast (150 g.) was added to 300 ml. boiling water, the mixture kept at boiling point for 3 min., cooled rapidly, and centrifuged. The 220 ml. supernatant were stored at $-10°$.

Chemicals. Succinic acid was crystallized twice from water, and the sodium salt prepared from this was crystallized twice from aqueous ethanol. α-Oxoglutaric acid and adenosine 5-phosphate (AMP) were commercial samples (Light and Co. Ltd.). Vitamin B_{12} was 'Cytamen' (Glaxo Laboratories Ltd.). We are grateful to Dr J. Judah for samples of glutathione, diphosphopyridine nucleotide and adenosine triphosphate (ATP), and to Lederle Laboratories (New York) for generous gifts of synthetic citrovorum factor (Leucovorin), folic acid and aminopterin. All other chemicals used were of A.R. grade. Solutions were made in glass-distilled water.

METHODS

Preparation of haemolysate. The blood was centrifuged at about 2000 rev./min., and the cells were washed twice with 0·9% NaCl at 2°. After the first washing, the leucocyte layer was removed by suction. The erythrocytes from 25 ml. blood were cooled to 0° and 21 ml. water at 0° added with hand-stirring. The stirring was continued and the mixture maintained between 0° and 1° for 5 min., when 5·25 ml. 0·15 M-KH_2PO_4/Na_2HPO_4 buffer, pH 7·4, containing 0·483 M-KCl, were added. These concentrations were calculated to restore approximate isotonicity.

Microscopic examination of this preparation, including the use of phase contrast, revealed free nuclei and ghosts, but no intact erythrocytes. The absence of the latter was confirmed by centrifugation at about 2000 rev./min.; under conditions chosen for incomplete haemolysis, remaining whole erythrocytes pack down as a dense, dark layer, but after haemolysis in the way described above, no trace of this dark layer is observed. The cell solids pack into a rather diffuse opaque layer under a clear red supernatant; the latter contains occasional fragments of ghost cells and clumps of minute granules of less than 0·5 μ. diameter, which can be shown up by phase contrast or by staining with Sudan black or pyronin. We are indebted to Dr G. Christie for the microscopic examinations. The following two preparations were used: (*A*) The haemolysate obtained by the above procedure (34 ml. approx.) was centrifuged at 2000 rev./min. and 2°, and sufficient clear supernatant removed to leave a total volume including the solids of 21 ml. (*B*) 21 ml. of the whole haemolysate. This corresponds, in terms of erythrocytes, to approximately 15·5 ml. of the original whole blood. The activity of this preparation was about 15% lower than that of preparation *A*.

Incubation. The haemolysate was placed in 100 ml. conical flasks plugged with cotton wool, and incubated with shaking at 90–100 shakes/min. at 38°. Each flask contained 21 ml. preparation *A* or *B*; additions were made so as to maintain approximate isotonicity, and the volume made up to 33 ml. with 0·122 M-KCl. When whole washed cells were incubated, 0·154 M-NaCl (0·9%, w/v) was used for making up to volume.

Isolation and purification of porphyrin. Protoporphyrin dimethyl ester and its Cu^{2+} *complex.* Protoporphyrin ester, mainly derived from the haem of haemoglobin (see discussion), was prepared by the method of Grinstein (1947) with two crystallizations from $CHCl_3$:methanol; further recrystallization did not alter the radioactivity. Crystalline protoporphyrin ester is often difficult to pack into planchets to give a smooth surface; preparation of the copper complex overcame this difficulty. For preparation of the Cu^{2+} complex, once-crystallized protoporphyrin ester (about 30 mg.) was dissolved in about 1 ml. hot pyridine, and about 0·5 ml. of a boiling, saturated solution of copper acetate in pyridine was added. The copper complex, which crystallized on cooling, was filtered at the pump and washed with methanol, boiling water and methanol again. It was found repeatedly that recrystallization of the Cu^{2+} complex from $CHCl_3$:methanol did not alter the radioactivity. For routine radioactivity determinations the crystalline Cu^{2+} complex prepared from once-crystallized porphyrin ester was used.

In one experiment, active glycine (10·6 μC) was added to the incubated haemolysate just before precipitation with acetone (cf. Grinstein, 1947). The resulting protoporphyrin ester, crystallized once only, had zero activity, showing that free glycine was completely removed during the Grinstein procedure. Further, when boiled haemolysate was incubated with glycine (12·7 μC), the isolated protoporphyrin ester (as the Cu^{2+} complex) had zero activity, as against 61 counts/min. in the unboiled control.

Determination of free (non-haem) prophyrins. Free copro- and proto-porphyrins were extracted essentially by the method of Schwartz & Wikoff (1952) and determined with the Beckman spectrophotometer.

Assay of radioactivity. Crystalline materials were packed in polythene planchets (1 cm.²), and the radioactivity was determined with a Geiger-Müller counter having a thin mica window. Counts were made at infinite thickness as determined experimentally for the various materials. Corrections for thickness were made where necessary, and standard errors calculated as described by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949).

Extraction of radioactive uncombined glycine from haemolysate. The protein of the haemolysate was precipitated with cold trichloroacetic acid (final concentration of acid 5%, w/v), and the supernatant obtained after centrifugation was passed through an anionic ion-exchange resin (De-acidite E). The eluate was treated with 1-fluoro-2:4-dinitrobenzene; the 2:4-dinitrophenylglycine was isolated by chromatography on Celite, essentially by the method of Perrone (1951), and crystallized from ethyl acetate-*cyclo*hexane.

Dilution of 2:4-dinitrophenylglycine for assay of radioactivity. Dinitrophenylglycine was determined spectrophotometrically at 360 mμ. in 0·5% (w/v) aqueous $NaHCO_3$ (Krol, 1952). Suitable samples of carrier and radioactive materials were then mixed, the solution was acidified and the dinitrophenylglycine returned to ether for crystallization. No appreciable hydrolysis occurred during the short exposure to alkali, since subsequent chromatography on Celite showed only one band, identified as 2:4-dinitrophenylglycine. Dilution of 2:4-dinitrophenylglycine of known radioactivity by this method gave the expected result.

Microbiological assays. Vitamin B_{12} was kindly assayed by Dr G. M. Ross, using *Euglena gracilis*; folic acid and citrovorum factor assays, with *Lactobacillus casei* and *Leuconostoc citrovorum*, respectively, were kindly performed by Dr J. Lascelles.

RESULTS

Effect of time of incubation and of glycine concentration. Fig. 1 illustrates the effect of time of incubation on the extent of incorporation of labelled glycine in haemolysates. In the presence of boiled yeast extract (see below), the synthesis fell off gradually until at 8 hr., it had practically ceased. Up to 4 hr., the mixture remained clear but after this time the formation of a gel became progressively greater. Both in the presence and absence of yeast extract the synthesis was virtually linear for 2 hr. Clearly, the amount of incorporation in 2 hr. serves as an approximate index of the initial rate of synthesis, and further effects were therefore studied on this basis.

The effect of glycine concentration on the initial rate of synthesis is shown in Fig. 2. Maximal activity was obtained with concentrations of added glycine above 0·015 M.

Endogenous glycine levels. The concentration of free glycine in a haemolysate was determined by measuring the extent of dilution of added radioactive glycine. All radioactivity measurements were made after conversion of glycine into its pure 2:4-dinitrophenyl derivative (see Methods section).

The initial endogenous glycine level in 21 ml. haemolysate *B* was found to be of the order of 3 mg. (Table 1, flask 1); after 2 hr. incubation there was no appreciable change in the apparent endogenous glycine, whether radioactive glycine was added at relatively low or high concentration (0·0013 M and 0·0065 M, cf. Fig. 2), as will be seen from flasks 2 and 3 (Table 1). Thus the radioactivity of available glycine remains virtually constant, at least during 2 hr. of incubation.

Recalculation of the results shown in Fig. 2, to allow for dilution by endogenous glycine, displaced the curve somewhat, but the saturation level was not altered appreciably.

Effect of variations in preparation on activity of haemolysates. Leucocytes were usually removed before haemolysis (see Methods); in one experiment, where they were allowed to remain, a 30% decrease in the glycine incorporation was found.

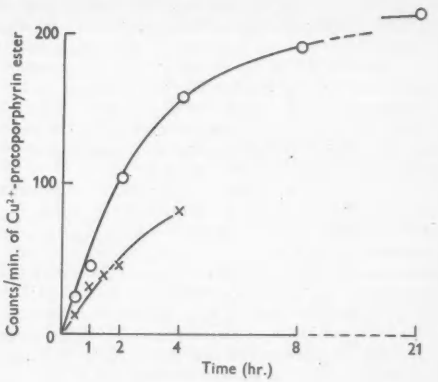

Fig. 1. Effect of time of incubation on incorporation of [α-^{14}C]glycine. ○—○, Haemolysate *A* (21 ml.) incubated at 38° with 0·0036 M-$MgCl_2$, 0·01 M sodium succinate, 0·0055 M sodium acetate, 0·00036 M AMP, 0·013 M glycine (8·54 μc) and 3·0 ml. boiled yeast extract; ×—×, haemolysate *B* (21 ml.) incubated with $MgCl_2$, sodium succinate and glycine as above.

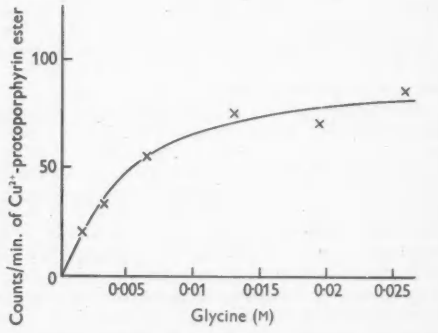

Fig. 2. Effect of concentration of glycine on its incorporation. Haemolysate *B* (21 ml.) incubated 2 hr. at 38° with 0·01 M sodium succinate, 0·0036 M-$MgCl_2$ and glycine (0·4 μc/mg.).

Table 1. *Dilution of added radioactive glycine by endogenous glycine*

(Flasks contained 21 ml. haemolysate *B*, 0·0036 M-$MgCl_2$ and 0·01 M sodium succinate in a final volume of 33 ml. The [α-^{14}C]glycine added had 570×10^3 counts/min./cm.^{2} at infinite thickness (0·801 μc/mg.). Flask 1 was treated immediately with trichloroacetic acid, and flasks 2 and 3 were treated similarly after incubation for 2 hr. at 38°. Glycine was isolated and its radioactivity determined as described under Methods.)

Flask	[α-^{14}C]Glycine added (mg.)	Time incubated (hr.)	Radioactivity of isolated glycine as % of added glycine	Calculated endogenous glycine (mg.)
1	3·2	0	57	2·41
2	3·2	2	51	3·07
3	16·0	2	85	2·82

Storage of the haemolysate at 2° for 24 hr. before incubation resulted in a loss of only 10% of the initial activity.

Different methods of restoring approximate isotonicity after haemolysis were investigated. The activities of haemolysates were similar, whether the water added for haemolysis was brought to a final concentration of 0·122M potassium chloride or 0·25M sucrose on the one hand, or 0·097M potassium chloride with 0·03M phosphate of pH 7·4, or 0·20M sucrose with 0·03M phosphate on the other. At the end of incubation, the pH of these haemolysates varied between 7·45 and 7·48, indicating that the buffering capacity of the system was sufficient even in the absence of added phosphate buffer.

Effect of various additions. Neither sodium succinate (0·01M) nor sodium α-oxoglutarate (0·01M), added separately or together, affected the incorporation of glycine significantly. The addition of sodium acetate (0·0055M) appeared to cause slight inhibition, but the purity of this sample was not studied (Table 2). No appreciable effect was obtained with magnesium chloride (0·0036M), ferrous sulphate (0·005M), or diphosphopyridine nucleotide (0·0005M) in the presence of 0·04M nicotinamide. AMP inhibited slightly, but the purified sample of ATP gave approximately 30% stimulation.

The effect of adding boiled yeast extract is shown in Table 3. Whereas some inhibition was found with intact washed erythrocytes, the synthesis in haemolysates was stimulated about sixfold at the optimum concentration of boiled yeast extract. With further increases in concentration the stimulation decreased, the yeast preparation apparently containing a substance which inhibited at higher concentrations. The effect was not entirely consistent, and with some preparations maximum stimulation was not more than twofold.

Table 2. *Effect of various additions on incorporation of* [α-^{14}C]*glycine in haemolysates*

(Control flasks contained 21 ml. haemolysate *A* or *B*, 0·0036M-$MgCl_2$, 0·01M sodium succinate, 0·0055M sodium acetate and glycine (0·0065M (6·4 μc) in Expts. 30 and 32, 0·0065M (12·8 μc) in Expt. 80 and 0·013M (12·8 μc) in Expts. 42 and 44). Incubation 2 hr. at 38°. Other conditions as described in Methods section.)

Expt. no. (Preparation)	Conditions	Counts/min. of Cu^{2+}-protoporphyrin ester	Activity as % of control (averages taken where necessary)
42 (*B*)	Control	56±2·9	100
	$MgCl_2$ omitted	48±2·7, 51±2·8	88
44 (*B*)	Control	40±2·7, 49±2·8	100
	Sodium acetate omitted	62±3·1	139
	Sodium succinate omitted	43±2·7	97
30 (*A*)	Control	77±3·2	100
	Control + AMP (0·00036M)	73±3·2	95
	Control + ATP (0·00085M)	99±3·6	129
32 (*A*)	Control	65±3·1	100
	Control + AMP (0·00036M)	46±2·8, 49±2·8	73
	Control + AMP + $FeSO_4$ (0·005M)	47±2·8	72
80 (*B*)	Control*	84±3·4, 91±3·5	100
	Control + α-oxoglutarate (0·01M)	80±3·3	91
	Control + α-oxoglutarate (0·01M), succinate omitted	94±3·5	107
	Control + diphosphopyridine nucleotide (0·0005M) + nicotinamide (0·04M)	87±3·4	99

* Sodium acetate was omitted throughout Expt. 80.

Table 3. *Effect of boiled yeast extract on incorporation of* [α-^{14}C]*glycine*

(Washed cells from 25 ml. blood made up to 25 ml. with 0·9% (w/v) NaCl, or 21 ml. haemolysate *A*, incubated 18 hr. at 38° with $MgCl_2$, sodium succinate and sodium acetate as in Table 2, and 0·0016M glycine (2·13 μc). Expt. 18 also contained 0·00036M AMP. Final volume was 33 ml.)

Expt. no.	Preparation	Counts/min. of Cu^{2+}-proto-porphyrin ester	Activity as % of control
14	Washed cells	353±3·8	100
	Washed cells + boiled yeast extract (1·0 ml.)	270±3·4	77
18	Haemolysate	24±2·3	100
	Haemolysate + boiled yeast extract (2·0 ml.)	105±3·7	438
	Haemolysate + boiled yeast extract (4·0 ml.)	135±4·1	563
	Haemolysate + boiled yeast extract (6·0 ml.)	80±3·3	334
	Boiled yeast extract alone (2·0 ml.)	0±1·7	0

2:4-Dinitrophenol (DNP) was added both to washed erythrocytes and to haemolysate preparations under conditions similar to those described in Table 3. In intact washed erythrocytes, the synthesis was inhibited 73% by 0·0004M DNP. In haemolysates, 78 and 93% inhibitions were found with 0·0001M and 0·0005M DNP, respectively.

Calcium chloride at 0·0002M inhibited 46% in haemolysates.

Changes in free porphyrin. Studies were undertaken of the changes in free (non-haem) porphyrin in haemolysates incubated with non-labelled glycine. The total free porphyrin, extractable from ethyl acetate with 10% (w/v) aqueous hydrochloric acid, increased on incubation for 2 hr. to about twice the initial level (Table 4). It was mainly protoporphyrin, coproporphyrin amounting to not more than 10%.

Effect of fluoride. Sodium fluoride was found by Gajdos & Gajdos-Török (1951) to cause a large increase in free protoporphyrin formation in blood from phenylhydrazine-anaemic rabbits *in vitro*. We have tested its effect on haem synthesis and on free porphyrin formation. Free porphyrins were extracted and determined as described under Methods, and the porphyrin-free haemin, which remained in the ethyl acetate solution, was converted to pure protoporphyrin ester copper complex for radioactivity determinations.

In haemolysates, haem synthesis was inhibited 33 and 48% by 0·02M and 0·04M sodium fluoride, respectively (Table 5). At the low concentrations found in haemolysates, free porphyrin determinations are subject to rather large experimental error. Thus small inhibitions cannot be detected with sufficient accuracy, and the values obtained with 0·02M and 0·04M sodium fluoride were probably not significantly different from the control values. With 0·12M sodium fluoride (the concentration used by Gajdos & Gajdos-Török, 1951) there was marked inhibition (Table 5).

Table 4. *Free porphyrin synthesis in haemolysates*

(21 ml. haemolysate *B* incubated 2 hr. at 38° with 0·013M glycine, 0·01M sodium succinate and 0·0036M-$MgCl_2$; Expts. 47 and 95 also contained 0·0055M sodium acetate.)

Expt. no.		No. of replicates	Free porphyrin as protoporphyrin (μg.) Mean	Range
95	Initial value	5	4·6	4·1–5·1
	After incubation	7	10·2	8·1–11·7
47	Initial value	2	5·2	4·0–6·3
	After incubation	2	10·6	10·2–11·0
54	Initial value	2	2·2	1·8–2·6
	After incubation	2	5·1	3·9–6·3

Table 5. *Effect of fluoride on haem and porphyrin synthesis*

(Whole blood: 25 ml. incubated 18 hr. at 37° with 0·055M-[α-^{14}C]glycine (10·68 μc), final vol. 30 ml. Haemolysates: in Expt. 32, 21 ml. haemolysate *A* incubated 2 hr. at 38° with 0·00036M AMP, as well as additions shown in Table 2; in Expt. 60, 21 ml. haemolysate *B* incubated 2 hr. at 38° with 0·01M sodium succinate and 0·013M glycine.)

Expt. no.	Sodium fluoride (M)	Counts/min of Cu^{2+}-protoporphyrin ester prepared from haem	% Inhibition of haem synthesis (averages taken where necessary)	Free porphyrin as protoporphyrin No. of replicates	Mean (μg.)	Range	% Inhibition of porphyrin synthesis
Whole blood							
60	—	125±3·9, 128±4·0	—	3	462	435–485	—
	0·12	40±2·6, 41±2·7	68	2	34	—	93
62	—	155±4·5	—	2	530	515–543	—
	0·12	37±2·6	76	2	66	61–72	88
Haemolysate							
32	—	46±2·8, 49±2·8	—	—	—	—	—
	0·02	32±2·5	33	—	—	—	—
	0·04	25±2·4	48	—	—	—	—
60	—	—	—	1	7·2	—	—*
	0·02	—	—	3	6·1	5·6–6·3	21
	0·04	—	—	3	6·3	4·2–7·7	17
	0·12	—	—	3	2·5	2·2–2·7	90

* No initial value for free protoporphyrin was determined, and a figure of 2 μg. is assumed from experience with other experiments.

In whole blood, fluoride again inhibited both free porphyrin and haem synthesis (Table 5). The fluoride inhibition of free porphyrin formation in normal chicken blood has been confirmed by Gajdos (personal communication), the behaviour of this tissue thus standing in direct contrast to that of blood from phenylhydrazine-anaemic rabbits.

Haemopoietic factors. Vitamin B_{12}, from 0·15 to 0·6 μg./ml., made no difference to the rate of incorporation of [α-^{14}C]glycine into the porphyrin nucleus in haemolysates. A similar result was found with haemolysates from the blood of B_{12}-deficient chicks and from control chicks fed supplementary B_{12} (Table 6). The blood of these chicks had similar synthetic activity to that of normal adult fowls.

Haemolysates from normal chicken erythrocytes contained no measurable B_{12} (i.e. less than 0·3 μmg./ml.). Added B_{12} (0·1 μg./ml.) was not broken down on incubation for 2 hr.

Folic acid, in relatively high concentrations, stimulated as shown in Fig. 3. The results of the two experiments shown here agreed well, but the effect was nevertheless variable, the stimulation at 0·002 M ranging in different experiments from 20 to 70%. Ascorbic acid caused 20% stimulation at 0·0057 M, but did not potentiate folic acid (Table 7). Reduced glutathione (0·001 M) had no effect.

Folinic acid, supplied as the calcium salt (Leucovorin) was found to be inhibitory, and it was found that calcium chloride at the same molarity (0·0002 M) inhibited to precisely the same extent (see above). Free folinic acid was prepared from Leucovorin after precipitating the calcium ions as calcium oxalate, and was shown by microbiological assay to have the same activity as the original Leucovorin. This material had no effect whatever on the incorporation of [α-^{14}C]glycine by haemolysates at concentrations from 1×10^{-6} M to 2×10^{-4} M.

Table 6. *Effect of vitamin B_{12} on incorporation of [α-^{14}C]glycine in haemolysates from B_{12}-deficient chick blood*

(For description of experimental material see Methods. 21 ml. haemolysate *B* incubated 2 hr. at 38° with 0·01 M sodium succinate, 0·0036 M-$MgCl_2$ and 0·013 M glycine (12·8 μc).)

	Counts/min. of Cu^{2+}-protoporphyrin ester	
	No addition	Added vitamin B_{12} (0·3 μg./ml.)
Blood from B_{12}-deficient chicks	74±3·5	77±3·5
Blood from control chicks (B_{12} added to diet)	60±2·7 56±3·2	53±3·2

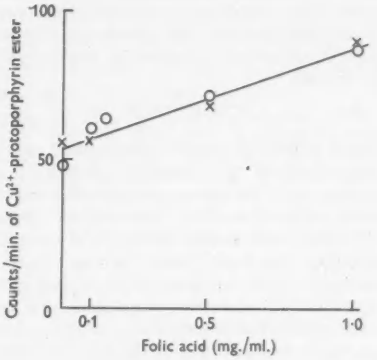

Fig. 3. Effect of folic acid on incorporation of [α-^{14}C]-glycine. Haemolysate *A* incubated under conditions described in Table 2, including 0·00036 M AMP and 0·0065 M glycine (6·4 μc). Results from two experiments indicated by (○) and (×).

Table 7. *Effect of folic acid, aminopterin and ascorbic acid, on incorporation of [α-^{14}C]glycine in haemolysates*

(21 ml. haemolysate *B* incubated 2 hr. at 38° with 0·01 M sodium succinate, 0·0036 M-$MgCl_2$ and 0·0065 M glycine (8·54 μc).)

Expt. no.	Additions	Counts/min. of copper protoporphyrin ester	Activity as % of control (averages taken where necessary)
56	None	63±3·0, 65±3·0	100
	Folic acid (0·002 M)	90±3·4	141
	Aminopterin (0·002 M)	83±3·3	130
	Folic acid (0·002 M) + aminopterin (0·002 M)	72±3·2	113
	Ascorbic acid (0·0057 M)	77±3·3	120
	Folic acid (0·002 M) + ascorbic acid (0·0057 M)	95±3·5	149
58	None	66±3·1, 69±3·2	100
	Folic acid (0·002 M)	81±3·4	120
	Folic acid (0·004 M)	80±3·4	119
	Aminopterin (0·002 M)	62±3·1	92
	Aminopterin (0·004 M)	43±2·8	64
	Folic acid (0·002 M) + aminopterin (0·002 M)	66±3·2	98

The folic acid content of haemolysates from normal chicken erythrocytes was 0·3 μmg./ml. (6×10^{-10}M); about 90% of this had disappeared after 2 hr. incubation. The folinic acid content of the haemolysate was below the limit of assay. After 2 hr. incubation, recoveries of added folic acid (0·002M) and folinic acid (0·0001M) were 70 and 30%, respectively.

Aminopterin, which is a specific antagonist of folic acid in some reactions, might have been expected to decrease the folic acid stimulation. It was found in one set of experiments that, where folic acid stimulated 40%, aminopterin at the same concentration (0·002M) stimulated 30%; when both were added together, 13% stimulation was found. In another experiment, 0·002M aminopterin alone caused 8% inhibition, increasing to 30% at 0·004M (Table 7). The effects with aminopterin were thus very variable, and possible interpretations of the findings with this and the pterin factors are discussed below.

DISCUSSION

The study presented here is a preliminary survey of the properties of the tissue preparation; these haemolysates of chicken erythrocytes retain a considerable proportion of the capacity of the whole cells for haem synthesis. Perhaps the most interesting effect we have found is the remarkable stimulation by some samples of boiled yeast extract. We have not yet identified the responsible factor, but we calculate that at optimum boiled yeast extract concentration the activity of the haemolysate was approximately 80% of that of whole cells. It is interesting to note the finding of Rimington (1943), that the formation of coproporphyrin during incubation of fresh baker's or brewer's yeast is stimulated by a boiled, concentrated extract of baker's yeast.

The radioactivity of protoporphyrin ester isolated by the procedure of Grinstein (1947) has been used extensively, in the experiments reported here, as an index of haem synthesis. The method does, in fact, measure mainly haem synthesis, but a small amount of the free porphyrin synthesized is also included. For critical comparison of haem and free porphyrin synthesis, a method such as that described in the experiment on the effect of fluoride (above) must be used. In a subsequent paper such methods of differential analysis will be discussed in detail.

The free porphyrin formed in our haemolysates during 2 hr. incubation amounted to 2–5 μg./15 ml. blood. In this range our experimental error was too large to allow a detailed study of the effects of inhibitors, and attempts to elucidate the effect of lead on haemopoiesis were unsuccessful. It could be shown that the synthesis of both haem and free porphyrin was almost totally inhibited at 1×10^{-4}M lead acetate. At 1×10^{-5}M lead acetate, haem synthesis was inhibited by about 50%. It would be of interest to know whether free porphyrin synthesis was inhibited to the same extent (cf. Watson, 1950), but we were unable to decide this point because of the inaccuracy of the results. Attention must be drawn to an erroneous statement in our earlier publication (Falk & Dresel, 1952). The apparent stimulation of free porphyrin formation by lead observed in preliminary experiments was later shown to have been due to an artifact, as indeed was stated when the paper was read.

The results obtained on the addition of vitamin B_{12} make it quite clear that this has no effect on haem synthesis in haemolysates of erythrocytes from either normal or B_{12}-deficient birds. The effects found with folic acid and the other pterin compounds are much more difficult to interpret. It appears that in some tissues folic acid is converted to folinic acid (citrovorum factor), which is the metabolically active factor; this conversion has been reported to be accelerated by ascorbic acid and to be inhibited by aminopterin (cf. Welch & Heinle, 1951). In our experiments, haem synthesis was stimulated by folic acid at relatively very high concentrations, but this stimulation was not increased by ascorbic acid. Aminopterin, when added with folic acid, inhibited the stimulation due to the latter, but aminopterin added alone caused quite significant stimulation in some experiments. The effect of aminopterin was variable, however, and in other experiments inhibition to below the control level was found. While the inhibition by aminopterin of the effect of added folic acid speaks in favour of the folic stimulation being due to its primary conversion to a substance either identical to, or related to folinic acid, there is a good deal of evidence against such an interpretation. The concentrations of both folic acid and aminopterin necessary to show marked effects were relatively very high, ascorbic acid did not potentiate folic acid, folinic acid did not stimulate at all, and lastly, aminopterin added alone was found to stimulate on a number of occasions.

We cannot offer a satisfactory explanation of these effects, but feel that they are artifacts, rather than suggestive of a coenzyme requirement met by any of the pterin factors. There is, of course, no certainty that the endogenous concentrations of these factors are limiting in our system, and it is possible that such requirements might show up in a more sensitive preparation. However, we incline to the view that the increased haemoglobin formation caused *in vivo* by vitamin B_{12} and folic acid (cf. Welch & Heinle, 1951) is the simple consequence of the generation of more cells capable of producing haemoglobin, and is not due to direct stimulation of the synthesis of the haemoglobin molecule.

SUMMARY

1. A preparation of completely haemolysed erythrocytes from the blood of domestic fowls, which actively incorporates [α-^{14}C]glycine into haem, is described.

2. The effects of time and of glycine concentration on the rate of synthesis were studied.

3. Stimulation of the synthesis up to sixfold was obtained with some preparations of boiled yeast extract. 2:4-Dinitrophenol inhibited the synthesis almost completely at 5×10^{-4}M.

4. Synthesis of free protoporphyrin also occurs during incubation of the haemolysate.

5. Contrary to the findings of Gajdos & Gajdos-Török (1951), using the blood of phenylhydrazine-anaemic rabbits, sodium fluoride was found to inhibit free porphyrin synthesis both in haemolysates and in whole blood of fowls.

6. The effects of various other substances, including some erythropoietic agents, are also described.

We thank Prof. C. Rimington for his continuous encouragement and support, and Dr A. Neuberger, F.R.S., for the interest he has taken in this work. We have much pleasure in acknowledging the able technical assistance of Miss A. Benson throughout, and are grateful to B. Lewis and Co., for their kindness in providing generous supplies of chicken blood.

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Synthesis of Cellulose by *Acetobacter xylinum*

1. MICROMETHOD FOR THE DETERMINATION OF CELLULOSES*

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The use of micro-organisms in studies of cellulose synthesis is dependent on the development of suitable methods for the quantitative determination of cellulose in small samples. For this purpose, several types of approach might be considered; more especially, methods based on: (i) the colour reactions shown by carbohydrate on heating with a chromogen in the presence of strong acid, (ii) total combustion, and (iii) reductometry of a hydrolysate. In view of the relative specificity and precision of reductometric methods of sugar assay, it seemed preferable in principle to base the micromethod for cellulose on the last of the general possibilities listed above. However, the direct quantitative conversion of cellulose into glucose by acid hydrolysis under conditions tolerated by glucose requires many hours (cf. Heuser, 1946). It will be shown in this communication that the difficulty inherent in the direct hydrolysis of cellulose fibre can be avoided if the material is first subjected to acetolytic degradation. With this means the fibre is rapidly made soluble and a product is obtained which can be hydrolysed quantitatively with relative ease. A microprocedure for the estimation of cellulose has been based on this principle.

The method detailed below has been applied extensively only to the study of the formation of cellulose by washed cell suspensions of *Acetobacter xylinum* in glucose solution (Hestrin, Aschner & Mager, 1947). It is anticipated that the procedure will also prove suitable for use with other celluloses.

* Experiments presented in this communication were performed by one of us (M.S.) in part fulfilment of requirements for a Ph.D. degree.

It may have advantages, moreover, for determination of certain of the difficulty hydrolysable non-cellulosic polysaccharides, e.g. dextrans. The applicability to cotton cellulose as well as to bacterial cellulose has been verified.

MATERIALS AND METHOD

Reagents

Sodium hydroxide, 8 and 16 % (w/v).

Talcum powder.

Acetolytic reagent. Acetic anhydride, glacial acetic acid and sulphuric acid, sp.gr. 1·84 (1 : 1 : 0·05, v/v). This reagent is prepared immediately before use.

Hydrolytic reagent. Sulphuric acid (sp.gr. 1·84) and water (15 : 85, v/v).

Copper reagent. The highly alkaline copper reagent of Somogyi (1945) was used with a minor modification. To avoid autoreduction during storage, the $CuSO_4$ is stored separately in aqueous solution and added to the stock solution of the other required components on the day the reagent is used.

Arsenomolybdate solution. This was prepared as described by Nelson (1944).

Sulphuric acid reagent. Sulphuric acid (sp.gr. 1·84) and water (21 : 479, v/v).

Reference sample. Samples of pellicles from 7-day glucose cultures were rinsed, pressed out repeatedly in water, and treated with 1 % (w/v) NaOH at room temperature for 1 hr. and at 98° for 5 min. to dissolve the bacterial cells. The product, after further repeated rinsing and pressing in water, was dried *in vacuo* at 90°. (Found: ash, 1·0; N (by Kjehldal), 0·3, moisture, 3·6 %.)

Analytical procedure

Preparation of sample for analysis. The glassware should be scrupulously protected from contamination by airborne cellulosic dusts. The suspension of cells of *A. xylinum* with the formed cellulose floccules and pellicle is washed into a centrifuge tube which has a mark at 10 ml. The insoluble particles are packed by centrifuging (about 4000 rev./min. for 15 min.). Since, at this stage, the cellulose is accompanied by the cells which it enmeshes, it settles readily in the centrifugal field. In absence of weighting protein, or if entrained air bubbles are present, sedimentation may be facilitated by addition of a pinch of talcum powder and/or a little ether. The precipitate is freed from the water-soluble residues by resuspension in water and again packed by centrifuging. To the precipitate is added 1 ml. of water. Large clumps are broken by shaking. To the suspension, 1 ml. of 8 % (w/v) NaOH solution is added to give a final concentration of 4 %. To make the cells soluble, the suspension is held in a boiling-water bath for 5 min., the tube being shaken briefly after 2 or 3 min. to facilitate dispersion of the solids. Since the alkali-purified cellulose cannot be sedimented readily, a pinch of talcum powder is added to provide an effective weighting agent. In rare instances in which trapped air bubbles cause flotation, a glass rod shaken in the suspension serves to dislodge the troublesome bubbles. The volume is increased by addition of water, and the cellulosic material with the enmeshed talcum powder is packed tightly by centrifuging (about 6000 rev./min. for 5 min.). To remove residual alkali-soluble material and to lower the NaOH concentration, the precipitate is suspended in water and again packed by centrifuging. Finally, the tube is thoroughly drained and held at 80–90° until the material appears to be dry.

Determination of the cellulose content of the purified cellulosic fraction. The analytical procedure comprises three steps: solution of the purified fraction by acetolysis, acid hydrolysis of the product so obtained, and estimation of the liberated reducing sugar.

Acetolysis. To the dried and purified cellulosic fraction (50–5000 μg.) contained in a tube with a mark at 10 ml. is added 1 ml. of the acetolytic reagent. If the sample contains less than 50 μg. cellulose, use of a smaller volume of the acetolysing reagent is advantageous. The volumes of reagents added subsequently must then be reduced proportionately. The tube is covered with a glass bulb, transferred to a boiling-water bath, and shaken briefly at the beginning of the reaction to facilitate solution. After 20 min., the tube is removed from the bath and allowed to cool. A colourless or faintly amber solution is obtained. The particles of talcum powder do not need to be removed at this stage.

Hydrolysis. To the acetolysed cellulose solution (1 ml.) is added 1 ml. of the hydrolytic reagent and the fluids are mixed by shaking. The solution may become turbid at this stage if the cellulose content is high. The hydrolysis is conducted under a glass bulb for 40 min. in a boiling-water bath. The tube is then transferred to an ice bath and the contents are neutralized by addition of a suitable volume of 16 % NaOH solution. The volume of alkali solution required is determined beforehand on a blank which contains the same reagents without cellulose, phenolphthalein serving as the indicator. A small excess of alkali is difficult to avoid, but the loss of sugar from this cause is negligible when the temperature is kept low by the use of an ice bath. The solutions, immediately on the removal of each tube from the ice bath, are brought to the mark (10 ml.) by addition of water and mixed thoroughly with the aid of a pipette. The talcum powder present is removed. The reducing power is determined on a suitable sample not later than 1 hr. after completion of the hydrolysis.

Determination of reducing power. The copper reagent (2 ml.) and the sample of the cellulose hydrolysate are mixed, brought to a volume of 4 ml., and heated for 25 min. in a boiling-water bath. To the cooled solution is added 2 ml. of the arsenomolybdate solution, followed by sufficient of the sulphuric acid reagent (1 ml. reagent/ml. sample) to overcome the buffering power of the sodium acetate present in the sample. The solution is swirled, made up after 5 min. with water to 10 ml. and the colour read. Filter 54 (540 mμ.) is most satisfactory in the Klett photoelectric colorimeter. Filter 66 (660 mμ.) affords increased sensitivity and can be used when very small samples are to be determined. The measuring instrument is set to zero against a reagent blank which has been taken through the same procedure.

The values are read from a standard calibration curve obtained with glucose in water using the standard conditions. With a Klett photoelectric colorimeter as the measuring instrument, the lower threshold of the method is about 20 μg. The readings taken from the glucose calibration curve correspond without further correction to cellulose. Since 162 g. cellulose are equivalent to 180 g. glucose, the weight of glucose found should be multiplied by 0·9. However, as mentioned below, only 90 % of the bacterial or cotton cellulose is recovered as glucose. Consequently, when corrected for this loss, the overall conversion factor of glucose to cellulose is unity.

EXPERIMENTAL AND RESULTS

Alkali purification of the samples of bacterial cellulose

An alkaline reagent was desirable which would remove the non-cellulosic components promptly and with high degree of selectivity. It was found that treatment for 5 min. in 4% (w/v) sodium hydroxide at 97° fulfils this requirement. The insolubility of the cellulose of *A. xylinum* under these conditions was demonstrated by the finding that the analytical yield of cellulose was not increased when the concentration of sodium hydroxide was decreased from 4 to 1%.

Course of hydrolysis of acetolysed cellulose

The marked effect of primary acetolytic degradation on the course of the subsequent acid hydrolysis of the cellulose is shown by the experiment summarized in Fig. 1. When the preliminary acetolytic treatment was omitted, the recovery of reducing sugar even after 4 hr. hydrolysis was only about 5%. It is apparent that the hydrolysis of the acetolysed cellulose is completed under standard conditions within about 40 min., that the yield of reducing sugar at that time does not depend critically on the hydrolysis time, and that the recovery corresponds to 90% of the theoretical glucose yield. The shape of the hydrolysis curve of the acetolysed cellulose, and the observation reported later that glucose slowly loses reducing power under these conditions do not encourage the hope that a longer hydrolysis time would lead to an increased yield of reducing sugar beyond the observed maximum value.

An experiment to check whether the percentage yield of the reducing sugar under the conditions of the analysis is variable with sample weight is shown in Fig. 2. The points for both the *A. xylinum* cellulose and raw cotton cellulose fall on the same straight line which passes through the origin. The percentage yield of sugar is clearly independent of the weight of the cellulose sample within a wide range.

Applicability of the procedure to celluloses from different sources

It has been shown that the method is applicable to bacterial celluloses, both to the cellulose formed by *A. acetigenum* and to that formed by *A. xylinum*. The applicability to cotton cellulose has been verified with raw cotton samples taken from two locations and on a sample of cotton yarn containing 95% α-cellulose and 5% γ-cellulose. The recovery of reducing sugar under standard conditions in the cases listed above averaged 90% of the theoretical, the scatter of the results around this mean being within the range ±4%. In four analyses on filter paper, the recovery of glucose was 92±4%.

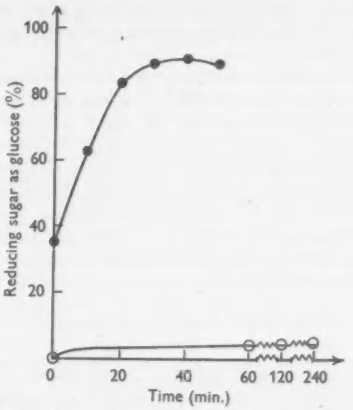

Fig. 1. Hydrolysis of bacterial cellulose. Samples of bacterial cellulose (~5 mg.) were weighed on the semi-microbalance. Reagent compositions and temperatures as described for the standard procedure. ●, cellulose after previous acetolysis; hydrolysis reagent added at zero min. ○, cellulose without previous acetolysis; hydrolysis reagent and acetolysis reagent added simultaneously at zero min.

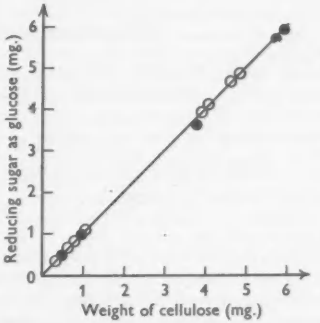

Fig. 2. Influence of weight of cellulose on sugar recovery. Samples of purified fibre were weighed on a semi-microbalance accurate to 0·02 mg. Cellulose weights are corrected for moisture, ash and protein. Standard analytical procedure. ●, raw cotton (Egyptian); ○, *A. xylinum* cellulose.

When a sample of pure glucose was taken through the procedures described for the cellulose analysis, the reducing power was 80% of the original value, this value being independent of the duration of heating in the hydrolysis reagent between 0 and 50 min. The observed disparity between the behaviour of glucose and cellulose may be due to the presence of a free reducing group in the glucose. When glucose was exposed directly to the action of the hydrolysis reagent in the present of the acetylating reagent, a slow loss of reducing power occurred

with continued heating, the reducing power after 0, 40 and 50 min. of heating being 100, 91 and 89% respectively.

The findings with filter paper and glucose suggest that the conditions of the analysis, as adapted to bacterial cellulose, can be used with cellulose polymers of widely varying degree of polymerization. Although some loss of reducing power due to the instability of a free reducing group may be expected when the analysis is applied to the lower homologues, it is evident that, even in the extreme case of glucose, the overall loss of reducing power is not greater than 20%.

Influence of salt concentration on colour intensity

The neutralized cellulose hydrolysate contains approximately 1·7 m-moles/ml. sodium acetate and 0·3 m-mole/ml. sodium sulphate. The colour formed with the arsenomolybdate reagent was found to be unaffected by the presence of these salts, provided that the buffering action of the sodium acetate was overcome by an appropriate addition of acid.

SUMMARY

1. A technique for the purification and quantitative recovery of small samples of bacterial cellulose has been detailed.

2. A micromethod for cellulose has been based on the dissolution of the purified polysaccharide by acetolytic degradation, the conversion of the product into reducing hexose by acid hydrolysis, and the colorimetric estimation of the formed sugar with the aid of a copper reagent.

3. The applicability of the technique to bacterial celluloses and cotton cellulose has been verified.

4. The method is relatively rapid and specific and is appropriate to serial analysis. The lower threshold is 20 μg.; the error range is ± 4%.

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Biochemical Studies of Toxic Agents

5. OBSERVATIONS ON THE FATE OF ^{35}S-LABELLED ARYLSULPHURIC ACIDS FOLLOWING THEIR ADMINISTRATION TO THE RAT

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A number of workers (for example, Rhode, 1923; Sperber, 1948; Garton & Williams, 1949) have studied the fate of arylsulphuric acids in the animal body, and their results suggest that when these compounds are administered to animals almost the whole of the dose is excreted unchanged in the urine. These findings were based on the determination of the increase in ethereal sulphate excretion over the 'normal level' following the administration of the sulphuric acid derivative. As ethereal sulphate excretion fluctuates somewhat under normal conditions, it is clear that experiments of this type cannot be expected to reveal with certainty a small degree of hydrolysis of an arylsulphuric acid *in vivo*. A more satisfactory method for studying this problem is provided by the measurement of the excretion of ^{35}S-labelled inorganic sulphate following the administration of an arylsulphuric acid labelled with ^{35}S. This method has been used in the present investigation. The potassium salts of [^{35}S]phenylsulphuric acid, and [^{35}S]1- and [^{35}S]2-naphthylsulphuric acid were synthesized and administered to rats by stomach tube and by intraperitoneal injection. The urinary excretions of inorganic sulphate-^{35}S and total sulphate-^{35}S were determined at various times during the 96 hr. after dosing. Similar results were obtained with the three compounds, namely, that although most of the administered arylsulphuric acid was quickly excreted unchanged, some breakdown of the compound did occur in the organism.

EXPERIMENTAL

Synthesis of the potassium salts of phenylsulphuric acid and 1- and 2-naphthylsulphuric acid labelled with ^{35}S

The method used for the synthesis of the radioactive arylsulphuric acids was based on that described by Burkhardt & Lapworth (1926). Except in the final stages, the same procedure was used for the preparation of all three compounds. The [^{35}S]chlorosulphonic acid used in the syntheses was obtained from the Radiochemical Centre, Amersham. It was

packaged in glass vials each containing 1 ml., and at the time of its receipt its specific activity was 1·06 mc/ml. The hazardous stages of the synthesis were carried out in glove boxes.

CS_2 (20 ml.) and dimethylaniline (14·5 ml.) were stirred together in a wide tube cooled in ice water. Radioactive chlorosulphonic acid (2·0 ml.), containing about 1 mc ^{35}S/ml., was added slowly to the mixture. The reaction vessel was then removed from the ice water and the theoretical quantity of the phenolic compound (2·8 g. phenol, 4·3 g. 1- or 2-naphthol) was added with stirring. Stirring was continued for 1 hr. and the mixture was then allowed to stand overnight. The potassium salt of the arylsulphuric acid was formed by slowly adding 50% (w/v) aqueous KOH with continuous stirring and cooling until all the dimethylaniline had been liberated and the mixture was alkaline (about 12 ml. of the KOH soln. were required). The potassium salt separated and as much as possible of the liberated dimethylaniline was poured off. The remainder was removed by washing the potassium salt with three 100 ml. portions of ether. The potassium salt was then dissolved in the minimum quantity of water at 70–80° and the hot solution was filtered rapidly. The filtrate was allowed to cool to room temperature and then transferred to the refrigerator. The crystals which separated were filtered off, sucked dry on the filter and then washed with ether.

Potassium phenylsulphate and potassium 2-naphthylsulphate were readily purified by repeated crystallization from water. The potassium 1-naphthylsulphate was contaminated with 1-naphthol and it was purified by converting it into the *p*-toluidine salt by the procedure described by Barton & Young (1943). The *p*-toluidine salt was dissolved in hot water and treated with charcoal. The hot solution was filtered and the filtrate was treated with 20% (w/v) aqueous KOH to decompose the *p*-toluidine salt. The crystals of potassium 1-naphthylsulphate which separated on cooling were recrystallized from water.

After three crystallizations from water each of the three compounds was found to be free from inorganic sulphate, chloride and phenol (or naphthol). Further recrystallization of the compounds had no significant effect on their specific activity. Analysis of the compounds for S by the Carius method gave: potassium phenylsulphate, 15·4; calc. for $C_6H_5O_4SK$, 15·1%; potassium 1-naphthylsulphate, 11·8; potassium 2-naphthylsulphate, 12·2; calc. for $C_{10}H_7O_4SK$, 12·2%.

Measurement of ^{35}S

Radioactive sulphate was separated in the form of benzidine sulphate preparatory to the measurement of its ^{35}S content. Although detailed studies of this method of measuring ^{35}S have already been reported (Henriques, Kistiakowsky, Margnetti & Schneider, 1946; Young, Edson & McCarter, 1949), it was necessary to modify these procedures in order to meet the requirements of the present investigation.

^{35}S *content of solutions containing radioactive inorganic sulphate.* The inorganic sulphate content of the solution was adjusted by dilution or by the addition of non-radioactive Na_2SO_4 solution so that 6·0 ml. of the solution contained not less than 2 mg. of inorganic sulphate-S, and preferably not more than 3 mg. To this solution, in a lipped boiling tube, were added 4·0 ml. of 95% (v/v) ethanol followed by 2·0 ml. of benzidine hydrochloride reagent (prepared as described by Young *et al.* 1949). The contents of the tube were then gently mixed and allowed to stand for 30 min. before filtration. The precipitate of benzidine sulphate was separated using an apparatus of the type described by Henriques *et al.* (1946) in which the filter paper rests on a sintered-glass plate. Whatman no. 30 filter papers of 2·3 cm. diameter were used for the filtration. The boiling tube, filter and precipitate were washed with three 5 ml. portions of 95% (v/v) ethanol. As soon as filtration was complete the disk of filter paper carrying the precipitate was mounted on a carrier which consisted of two Perspex plates held together by three brass bolts. The dimensions of the lower plate were such that it fitted the rack of the chamber of the lead castle. The upper plate was somewhat smaller. The filter-paper disk carrying the radioactive benzidine sulphate was placed in the centre of the lower plate. The upper plate, in the centre of which was a circular hole 2·1 cm. in diameter, was then bolted to the lower plate so that the filter paper was held around its edge and the precipitate was left exposed. After it had been mounted on the carrier, the benzidine sulphate was dried in a desiccator over anhydrous $CaCl_2$ for at least 1 hr. before counting. The thickness of the window of the Geiger-Müller tube employed in the counting equipment was 1·8 mg./sq.cm. (EHM 2, General Electric Co., Ltd., London).

After each sample of radioactive benzidine sulphate had been counted, it was transferred to a boiling tube with 5·0 ml. of water and titrated with 0·02 N-NaOH using phenol red as indicator. By this means the sulphate-S content of the sample was determined, thus enabling a correction for self-absorption to be applied to the radioactivity measurement.

^{35}S *contents of the inorganic sulphate and total sulphate fractions of urine.* When the benzidine precipitation procedure was applied to a solution containing Na_2SO_4 and a radioactive arylsulphate, a precipitate was obtained which contained a significant proportion of the radioactivity originally present in the solution. It appeared, therefore, that ethereal sulphate had been precipitated together with the inorganic sulphate on the addition of the benzidine reagent. This was not unexpected in view of the known low solubility of organic base salts of arylsulphuric acids (cf. Barton & Young, 1943; Laughland & Young, 1944; Berenbom & Young, 1951). The radioactive material in the benzidine sulphate precipitate could not be removed by various washing procedures. Experiment showed, however, that when the benzidine sulphate precipitate was separated, dissolved and then reprecipitated, the amount of radioactive material contaminating the precipitate was greatly reduced and there was no significant loss of inorganic sulphate. This finding led to the development of the following reprecipitation procedure for the measurement of the ^{35}S content of the inorganic sulphate fraction of a solution containing radioactive ethereal sulphate.

A measured volume (less than 6·0 ml.) of the solution to be analysed was transferred to a 15 ml. tapered centrifuge tube and its inorganic sulphate-S content was adjusted to 2–3 mg. The volume of the solution was brought to 6·0 ml. with water, and 4·0 ml. of 95% (v/v) ethanol were added followed by 2·0 ml. of benzidine reagent. The mixture was allowed to stand for 10 min. and was then centrifuged for 10 min. The supernatant liquid was discarded and the precipitate was suspended in 5 ml. of 95% (v/v) ethanol. It was centrifuged and the supernatant was again discarded. The precipitate was decomposed by warming it with 1·0 ml. of 0·2 N-NaOH. The volume of the solution was brought to 5·9 ml. by the addition of water, and 4·0 ml. of 95% (v/v) ethanol were added followed by 0·1 ml. of 2 N-HCl and 2·0 ml.

of benzidine reagent. Where only two precipitations of the benzidine sulphate were required, the second precipitate was filtered, washed, dried, and counted by the procedure already described. Where three precipitations of the benzidine sulphate were required, the second precipitate was washed in the centrifuge tube, dissolved in NaOH sol., and reprecipitated. The benzidine sulphate was then filtered, washed, and counted in the usual way.

The effect of this reprecipitation procedure in reducing the amount of arylsulphate carried down with the benzidine sulphate was studied quantitatively. A series of experiments was carried out in which 2·0 mg. of inorganic sulphate-S were precipitated once in the presence of 0·01, 0·05, 0·10, 0·15, 0·20 and 0·25 mg. radioactive ethereal sulphate-S (as phenylsulphate, 1-naphthylsulphate or 2-naphthylsulphate) and the radioactivity of the precipitates was measured in the usual way. This series of experiments was then repeated using the same amounts of inorganic sulphate and radioactive ethereal sulphate, but instead of measuring the radioactivity of the first precipitate of benzidine sulphate, this precipitate was dissolved and precipitated a second time (phenylsulphate) and a third time (1- and 2-naphthylsulphate) by the methods already described. All determinations were carried out in duplicate, and Table 1 shows the mean values for experiments with solutions of inorganic sulphate and radioactive arylsulphate in water and in normal rat urine (2 ml.). It will be noted that two precipitations in the case of solutions containing phenylsulphate and three precipitations in the case of solutions containing 1- or 2-naphthylsulphate reduced the radioactivity of the final benzidine sulphate precipitate to less than 2% of the total radioactivity originally present as arylsulphate.

In the measurement of the ^{35}S content of the inorganic sulphate fraction of the urine the volume of urine used was between 1·0 and 5·0 ml., the amount used being dependent on its radioactivity. In each determination 2·0 mg. of sulphate-S (as Na_2SO_4) were added so that the inorganic sulphate-S was between 2·0 and 3·0 mg. Reprecipitation of the benzidine sulphate was carried out as already described.

The following method was used for the measurement of the ^{35}S content of the total sulphate fraction of the urine. A measured volume of urine was made N with respect to its content of HCl and 2·0 mg. of sulphate-S (as Na_2SO_4) were added. The urine was evaporated to dryness and the residue was taken up in 6·0 ml. of water, filtered if necessary, and 5·0 ml. of the solution was analysed for its ^{35}S content.

Total ^{35}S content of faeces. The faeces were ground in a mortar, and a weighed sample was oxidized by the Carius method as described by Young *et al.* (1949). The sulphuric acid produced by this procedure was precipitated as the benzidine salt, the radioactivity of which was then measured.

Total ^{35}S content of compounds. The Carius oxidation procedure was used.

Observations on the hydrolysis of arylsulphates in urine

The presence of radioactive inorganic sulphate in the urine of rats dosed with [^{35}S]arylsulphates can only be used as evidence of the breakdown of ethereal sulphates in the organism if it can be shown that no significant amount of hydrolysis of these compounds occurs in the urine during the period between its excretion and the time it is analysed. This was studied as follows.

To 20 ml. of rat urine was added 0·05 m-mole of radioactive ethereal sulphate (phenylsulphate, 1- or 2-naphthylsulphate). Two 1·0 ml. portions were withdrawn immediately and analysed for the ^{35}S content of the inorganic sulphate fraction using the reprecipitation procedure already described. The urine was allowed to stand at room temperature and samples were taken for ^{35}S analysis at 24, 48 and 72 hr. No evidence was found of breakdown of ethereal sulphate during the first 24 hr. From 24 to 72 hr. some breakdown did occur, but this was quite small and was not significant in the present investigation since no collection period exceeded 24 hr. and the urines were analysed immediately after they had been collected. It is possible that the small liberation of sulphate which did occur in the urine on standing was the result of bacterial action, for a number of bacteria have been shown to possess arylsulphatase activity (Young, Morrison & Whitehead, 1952; Whitehead, Morrison & Young, 1952).

Table 1. *The influence of reprecipitation of benzidine sulphate on the amount of arylsulphate-^{35}S precipitated together with 2 mg. inorganic sulphate-S*

(For details, see text.)

	Percentage of arylsulphate-^{35}S precipitated											
	Potassium phenylsulphate				Potassium 1-naphthylsulphate				Potassium 2-naphthylsulphate			
	In water		In urine		In water		In urine		In water		In urine	
Arylsulphate-S (mg.)	1st ppt.	2nd ppt.	1st ppt.	2nd ppt.	1st ppt.	3rd ppt.	1st ppt.	3rd ppt.	1st ppt.	3rd ppt.	1st ppt.	3rd ppt.
0·01	3·5	0·8	2·8	1·0	12·9	1·2	8·4	1·0	25·6	2·0	28·2	1·6
0·05	3·5	0·7	3·1	0·8	8·6	1·2	7·8	1·1	19·9	0·8	19·0	0·9
0·10	3·1	0·7	2·9	0·7	6·8	1·1	7·0	1·1	15·4	0·6	15·6	0·8
0·15	2·7	0·7	3·1	0·6	6·4	1·4	5·9	1·2	14·7	0·5	15·3	0·7
0·20	2·5	0·7	2·5	0·7	5·8	1·2	5·3	1·1	11·3	0·4	12·4	0·6
0·25	2·5	0·7	2·5	0·7	5·5	1·2	5·7	1·1	11·5	0·3	13·0	0·4

Metabolic experiments

Two metabolic experiments were performed with each of the three ^{35}S-labelled compounds: potassium phenylsulphate, potassium 1-naphthylsulphate, and potassium 2-naphthylsulphate. In the first experiment the compound was given by intraperitoneal injection, and in the second it was given by stomach tube. Two male rats weighing between 140 and 200 g. were used in each experiment. They were housed in a metabolism cage which permitted the collection of urine separate from faeces and they received food and water *ad lib.* Each rat received a single dose of 0·05 m-mole of the potassium salt of the radioactive arylsulphate dissolved in water (0·5 ml. in the case of phenylsulphate and 1-naphthylsulphate, and 1·0 ml. in the case of 2-naphthylsulphate). The urine of the dosed animals was collected for the periods 0–6, 6–24, 24–48, 48–72 and 72–96 hr. After each collection the cages were washed down with distilled water, and the urine and washings were filtered through a cotton-wool plug and made up to 100 ml. The urine was analysed for inorganic sulphate-^{35}S and total sulphate-^{35}S. The results obtained are shown in Table 2. The faeces were collected and stored in the refrigerator. The total collection of faeces for each experiment was sampled and analysed for total ^{35}S.

In every experiment over 80% of the administered ^{35}S was excreted in the urine as total sulphate during the 96 hr. period after dosing. The amount of ^{35}S which appeared in the faeces during this period was small. After intraperitoneal injection of the compounds, the total ^{35}S content of the faeces corresponded to the following percentages of the ^{35}S administered: phenylsulphate, 0·2, 1-naphthylsulphate, 0·3, 2-naphthylsulphate, 2·1; the amounts found after administration of the compounds by stomach tube were 0·2, 5·6 and 3·1, respectively.

Irrespective of whether the arylsulphates were given by intraperitoneal injection or by stomach tube, there was a rapid excretion of ^{35}S in the ethereal sulphate fraction of the urine, indicating that the administered compounds were being excreted unchanged. Some hydrolysis of the compounds did occur in the organism, however, for in the 96 hr. after their intraperitoneal injection the ^{35}S excreted as inorganic sulphate in the urine corresponded to 3·5% of the phenylsulphate, 11·4% of the 1-naphthylsulphate and 8·1% of the 2-naphthylsulphate, and after administration of the compounds by stomach tube the values were 5·5, 11·8 and 8·4%, respectively.

DISCUSSION

The presence of radioactive inorganic sulphate in the urine following the administration of the salt of a [^{35}S]arylsulphuric acid can be accepted as evidence that breakdown of the arylsulphuric acid has occurred in the organism provided certain conditions obtain. One of these is that the administered compound should be free from radioactive inorganic sulphate. This was ensured in the present work by recrystallizing each compound just prior to its use in the metabolic experiments and by testing it for the

Table 2. *The distribution of* ^{35}S *in the inorganic, ethereal, and total sulphate fractions of the urine* ($I^{35}S$, $E^{35}S$ *and* $T^{35}S$) *of pairs of rats following the administration of potassium phenylsulphate, potassium* 1*-naphthylsulphate, and potassium* 2*-naphthylsulphate* (0·10 *m-mole per pair of rats*) *by intraperitoneal injection and by stomach tube*

Period after dosing (hr.)	Excretion of ^{35}S in urine (% of ^{35}S administered)*								
	Potassium phenylsulphate			Potassium 1-naphthylsulphate			Potassium 2-naphthylsulphate		
	$I^{35}S$	$E^{35}S$	$T^{35}S$	$I^{35}S$	$E^{35}S$	$T^{35}S$	$I^{35}S$	$E^{35}S$	$T^{35}S$
Administration by intraperitoneal injection									
0–6	1·1	83·3	84·4	3·7	66·0	69·7	2·5	67·8	70·3
6–24	1·8	5·1	6·9	6·5	9·3	15·8	4·5	12·0	16·5
24–48	0·3	0·9	1·2	0·9	0·7	1·6	0·8	1·4	2·2
48–72	0·2	0·3	0·5	0·2	0·2	0·4	0·2	0·4	0·6
72–96	0·1	0·4	0·5	0·1	0·1	0·2	0·1	0·2	0·3
0–96	3·5	90·0	93·5	11·4	76·3	87·7	8·1	81·8	89·9
Administration by stomach tube									
0–6	3·2	79·0	82·2	1·7	41·5	43·2	2·1	55·2	57·3
6–24	1·9	9·3	11·2	8·0	26·0	34·0	5·0	23·6	28·6
24–48	0·3	0·4	0·7	1·5	3·7	5·2	0·9	1·8	2·7
48–72	0·1	0·1	0·2	0·4	0·5	0·9	0·3	0·5	0·8
72–96	<0·1	<0·1	<0·1	0·2	0·3	0·5	0·1	0·2	0·3
0–96	5·5	88·8	94·3	11·8	72·0	83·8	8·4	81·3	89·7

* Counts/min. administered by intraperitoneal injection: potassium phenylsulphate, 221990; potassium 1-naphthylsulphate, 162400; potassium 2-naphthylsulphate, 262000. Counts/min. administered by stomach tube: potassium phenylsulphate, 221990; potassium 1-naphthylsulphate, 148900; potassium 2-naphthylsulphate, 248900.

presence of inorganic sulphate. It is also necessary to avoid hydrolysis of urinary radioactive ethereal sulphate during the period which elapses between the excretion of the urine and the taking of samples for analysis. Experiments in which each of the radioactive arylsulphates used in the metabolic studies was added to rat urine showed that liberation of inorganic sulphate from these ethereal sulphates in the urine could be disregarded as a source of error provided the collection period did not exceed 24 hr. and the urine was analysed immediately afterwards.

In the present work it was found that when non-radioactive inorganic sulphate is precipitated by the benzidine method from a solution containing ethereal [^{35}S]sulphate, the precipitate which forms is radioactive, and the radioactivity cannot be removed by ordinary washing procedures. This difficulty was largely overcome by separating the precipitate, dissolving it in alkali, and reprecipitating it with benzidine hydrochloride. As Table 1 shows, the reprecipitation procedure always reduced the error to the point at which not more than 2% of the radioactivity present in the urine as arylsulphate appeared in the inorganic sulphate fraction. In general the error introduced from this source was considerably less than 2%.

The amounts of radioactive inorganic sulphate found in the urine corresponded to 4–12% of the administered arylsulphates and it appears, therefore, that the urine contained inorganic sulphate arising from a breakdown of the arylsulphates within the organism. The results in Table 2 also show that, except in one experiment, over half the dose of each arylsulphate appeared unchanged in the urine in the 6 hr. period after dosing, and that in every case more than two-thirds of the dose was excreted unchanged in the urine in the first 24 hr. period after dosing. In view of the small amount of arylsulphate which underwent hydrolysis in the organism, it is noteworthy that Hanahan & Everett (1950) found that in the rat a large proportion of a dose of sodium oestrone [^{35}S]sulphate was hydrolysed. They also found that after the administration of radioactive sodium oestrone sulphate to rats by the intravenous, subcutaneous, and gastric routes, the amounts of ^{35}S which appeared in the faeces were not greatly different from those excreted in the urine. In the present work the amounts of ^{35}S in the faeces were small compared with those in the urine and none exceeded 6% of the ^{35}S administered as arylsulphate. These observations show that the arylsulphates studied were well absorbed when given by stomach tube. The amounts of radioactive inorganic sulphate excreted in the urine following the administration of the arylsulphates by stomach tube were similar to those present after these compounds had been given by intraperitoneal injection and it appears likely that little hydrolysis of the arylsulphates occurred in the gastro-intestinal tract.

SUMMARY

1. Potassium phenylsulphate, potassium 1-naphthylsulphate and potassium 2-naphthylsulphate labelled with radioactive sulphur (^{35}S) have been synthesized using [^{35}S]chlorosulphonic acid.

2. A study has been made of the conditions necessary for the accurate measurement of the amount of ^{35}S present as inorganic sulphate in urine containing [^{35}S]arylsulphate.

3. Experiments have been carried out in which the potassium salts of [^{35}S]phenylsulphuric acid, and [^{35}S]1- and [^{35}S]2-naphthylsulphuric acid have been administered to rats (0·05 m-mole per rat) by intraperitoneal injection and by stomach tube.

4. Under these conditions little hydrolysis of these arylsulphates occurred in the rat, for the total excretion of radioactive inorganic sulphate in the urine in the 96 hr. after dosing was only 4–12% of the ^{35}S administered as arylsulphate.

5. Most of the administered arylsulphate was excreted unchanged in the urine within a few hours of dosing. Less than 6% of the ^{35}S administered as arylsulphate appeared in the faeces.

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Levels of Enzymes of the Direct Oxidative Pathway of Carbohydrate Metabolism in Mammalian Tissues and Tumours

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In a previous publication from this laboratory (Dickens & Glock, 1951), data of a semi-quantitative nature were given on the distribution of enzymes of the direct oxidative pathway of carbohydrate metabolism in several mammalian tissues. Apart from this work, however, and the quantitative data of Horecker & Smyrniotis (1951) on the 6-phosphogluconate dehydrogenase activities of some acetone-dried tissues, the distribution of the enzymes of this pathway has not been systematically investigated in animal tissues. Following the development of quantitative methods of assay of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Glock & McLean, 1953) and of methods for quantitatively estimating the breakdown of ribose 5-phosphate, the enzyme activity levels in these consecutive stages of the direct oxidative pathway have been determined in a variety of normal mammalian tissues and tumours. The results are reported in the present paper.

MATERIALS AND METHODS

D-Glucose 6-phosphate (G 6-P), 6-phosphogluconate (6-PG) and D-ribose 5-phosphate (R 5-P). These were available as those preparations of the barium salts used previously (Glock & McLean, 1953).

Triphosphopyridine nucleotide (TPN). This was prepared and analysed as described previously (Glock & McLean, 1953). It contained 75% TPN and no diphosphopyridine nucleotide (DPN).

Preparation of tissue supernatants. All tissues were disintegrated in 9 parts of ice-cold isotonic KCl (0·15 M-KCl containing 8 ml. 0·02 M-$KHCO_3$/l.), either in a Potter glass homogenizer or in a Nelco homogenizer (Measuring and Scientific Equipment Ltd., London); centrifuging and dialysis of the supernatants were then carried out as before (Glock & McLean, 1953). In the case of erythrocytes, washed cells from 10 ml. heparinized blood were haemolysed and diluted with water up to 10 ml.; 1 part of haemolysate was treated with 9 parts of isotonic KCl and centrifuged and dialysed as above. Plasma was diluted with 9 vol. isotonic KCl and then treated similarly. Since tissue disintegration was carried out in isotonic solution, no corrections were necessary for the dehydrogenase activities of erythrocytes, the degree of haemolysis in all cases being negligible.

Estimation of the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Dehydrogenase activities of dialysed tissue supernatants were determined spectrophotometrically according to the method of Glock & McLean (1953) using an amount of enzyme solution which reduced TPN at a rate not exceeding 0·015 μmole/min. The reaction mixture consisted of 0·5 ml. 0·1 M-$MgCl_2$, 0·5 ml. 0·25 M glycylglycine buffer (pH 7·6 for G 6-P dehydrogenase and pH 7·6 and 9·0 for 6-PG dehydrogenase), usually 0·02–0·10 ml. tissue supernatant and 0·2 mg. TPN in a total volume of 2·4 ml. The reaction was started by the addition of 0·1 ml. 0·05 M substrate to both cells, the blank mixture not containing TPN.

Breakdown of ribose 5-phosphate. This was followed at 30° in a system containing 0·1 ml. 0·05 M R 5-P (Na salt), 0·5 ml. 0·25 M glycylglycine pH 7·6, 0·5 ml. 0·1 M-$MgCl_2$ and 0·2 ml. tissue supernatant in a total volume of 2·5 ml. After 10 and 20 min., 0·5 ml. digest was removed and introduced into 4·5 ml. 0·3 N-$HClO_4$. After spinning, pentose was determined by the orcinol method in 1 ml. portions of the supernatant as described previously (Glock, 1952). Incubated controls were included omitting the substrate. When necessary, pentose values were corrected for sedoheptulose interference, employing Dische's unpublished modification of the orcinol reaction. We are indebted to Dr Z. Dische for details of this method and also to Dr J. K. N. Jones for a sample of sedoheptulosan which was used as a standard.

Two pH activity curves for the breakdown of R 5-P by liver supernatants both in 0·05 M glycylglycine and in 0·01 M tris(hydroxymethyl)aminomethane ('Tris') are given in Fig. 1. The pH optimum in glycylglycine is at pH 7·6 and in 'Tris' at approx. pH 8·0. The effect of variation in enzyme concentration on the rate of breakdown of R 5-P by liver supernatants is shown in Fig. 2. The initial reaction rate is proportional to enzyme concentration, provided that not more than approx. 50% of the R 5-P is broken down.

The intracellular distribution of the enzymes concerned with the breakdown of R 5-P by liver suspensions was determined using the same fractional centrifugation procedure as was employed previously (Glock & McLean, 1953). As in the case of G 6-P and 6-PG dehydrogenases, all of this activity was located in the soluble fraction of liver suspensions.

Units of enzymic activity. A unit is defined as that quantity of enzyme which, in the case of G 6-P and 6-PG dehydrogenases, reduces 0·01 μmole TPN/min. at 20° (Glock & McLean, 1953), or in the case of R 5-P breakdown, splits 0·01 μmole R 5-P/min. at 20°. Temperature coefficients, experimentally determined between 20 and 30°, of 1·7 for G 6-P and 6-PG dehydrogenase activities (Glock & McLean, 1953) and of 1·5 for R 5-P breakdown, were used to convert experimental values obtained at room temperature or 30° into the corresponding values at 20°.

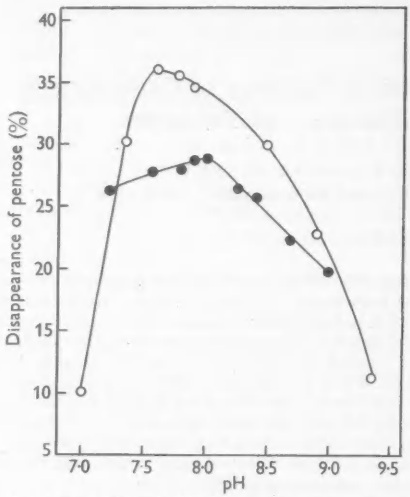

Fig. 1. Effect of pH on the breakdown of pentose phosphate by rat liver. Enzymic activity was determined, as described under 'Methods', on 0·2 ml. liver supernatant in 0·05 M glycylglycine buffer, ○—○; and in 0·01 M-tris-(hydroxymethyl)aminomethane ('Tris') buffer, ●—●.

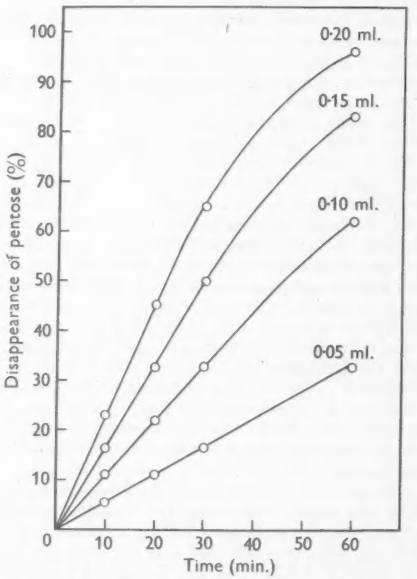

Fig. 2. Enzyme concentration/activity curves for pentose phosphate breakdown by rat liver. Enzyme activity was determined at 30° in 0·05 M glycylglycine buffer as described under 'Methods' with the indicated amounts of rat-liver supernatant.

Enzymic breakdown of TPN. At the end of the G 6-P dehydrogenase assay, Zwischenferment (G 6-P dehydrogenase; Kornberg, 1950) was added and the reduction of TPN followed to completion. In all cases, reduction was almost theoretical, indicating negligible breakdown of TPN by tissue supernatants at room temperature.

Phosphatase. Activity towards G 6-P, 6-PG and R 5-P was tested under the conditions of the assay procedures by following the liberation of inorganic phosphate. In no case was there more than 3% hydrolysis of substrate in 60 min. at 30°.

Resynthesis of hexose monophosphate. This was determined on the $HClO_4$ supernatants from the R 5-P breakdown experiments at the end of a 6 hr. incubation period, after neutralizing with KOH, cooling to 0°, and removal of $KClO_4$ by centrifuging (see Slater, 1953). Hexose monophosphate was estimated with Zwischenferment (Kornberg, 1950). As stated previously (Glock, 1952), our preparation of Zwischenferment (cf. Seegmiller & Horecker, 1951) contained isomerase, so that both G 6-P and F 6-P (fructose 6-phosphate) were estimated. The optimum concentration of Mg^{2+} ions, both for resynthesis of hexose monophosphate and for R 5-P breakdown was at a final concentration of 0·02 M, the concentration of Mg^{2+} used in the assay procedure.

RESULTS

Activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in tissues

Levels of dehydrogenase activities, expressed in units/g. tissue, in normal mammalian tissues are given in Tables 1 and 2. Since 6-PG dehydrogenase shows optimum activity at pH 9·0 (Glock & McLean, 1953), values for this dehydrogenase are given both at pH 7·6 and pH 9·0. High levels were found for both dehydrogenases in the adrenal glands, particularly in the adrenal cortex, in lymphatic tissue in general (spleen, thymus and lymph nodes), and in whole rat embryos in the early stages of development. In addition, the consistently high values found in the livers of adult female rats and in lactating mammary glands suggest that the levels of activity of both dehydrogenases are correlated with female sex hormones. Very low activities of both enzymes were found in skeletal muscle.

The results for mammary gland tissue in different physiological states of activity (Table 2) are of particular interest. The levels of activity of both dehydrogenases increase rapidly from the end of pregnancy to the end of lactation, this increase being approximately 60-fold for G 6-P dehydrogenase and 20-fold for 6-PG dehydrogenase, and then fall to very low levels in the involuting gland.

The results obtained with a variety of experimentally induced and spontaneous tumours are given in Table 3. All these values fall within the range of levels in normal tissues and are relatively high for the lymphomas and low for sarcomas.

Table 1. *Levels of enzymes of the direct oxidative pathway of carbohydrate metabolism in mammalian tissues*

(A unit of enzyme activity is defined as that quantity of enzyme which, in the case of G 6-P and 6-PG dehydrogenases, reduces 0·01 μmole TPN/min. at 20°, or, in the case of R 5-P breakdown, splits 0·01 μmole R 5-P/min. at 20°. Figures in parentheses are number of animals.)

		Enzyme activity (units/g. tissue at 20°)			
			6-PG dehydrogenase		
Tissue	Species*	G 6-P dehydrogenase	pH 9·0	pH 7·6	R 5-P breakdown
Adrenal gland	Rat (6)	163±25	350±28	185±9	425±29
Adrenal gland	Rabbit (1)	765	420	306	—
Adrenal cortex	Ox (2)	730	315	232	286
Adrenal medulla	Ox (2)	67	114	67	109
Spleen	Rat (4)	305±31	96±7	64±3	287±34
Spleen	Mouse (4)	106±16	84±8	47±3	393±53
Thymus	Rat (6)	98±1	87±9	49±5	359±14
Lymph nodes	Rat (1)	76	76	44	227
Liver	Adult female rat (12)	104±12	290±16	130±12	371±6
Liver	Adult male rat (18)	46±3	147±10	59±8	345±10
Liver	Young female rat (7)	55±9	122±8	62±4	232±17
Liver	Young male rat (7)	71±7	106±6	59±5	208±25
Lung	Rat (5)	88±6	106±7	59±3	282±44
Kidney	Rat (3)	69±7	62±5	56±3	259±9
Kidney cortex	Rabbit (1)	44	79	67	—
Kidney medulla	Rabbit (1)	32	39	27	—
Erythrocytes†	Rat (5)	40±2	34±1	18±0·4	212±11
Ovary	Cow (2)	30	52	35	108
Testis	Rat (4)	30±3	44±9	22±4	224±35
Prostate	Rat (6)	27±4	63±17	35±8	532±77
Seminal vesicles	Rat (4)	7±1	30±2	14±1	104±10
Placenta (20 days pregnancy)	Rat (1)	37	54	32	222
Brain	Rat (5)	32±3	22±1	11±1	75±3
Pituitary	Ox (3)	25	94	54	117
Duodenal mucosa	Rabbit (1)	20	54	58	—
Thyroid gland	Rabbit (1)	16	41	28	—
Cardiac muscle	Rat (6)	26±13	34±7	18±3	149±5
Skeletal muscle	Rat (6)	8±1	15±0·5	8±0·3	91±15
Skeletal muscle	Mouse (4)	19±5	25±8	15±4	209±49
Whole embryos					
10-day embryo	Rat (2)‡	186	140	71	430
20-day embryo	Rat (3)	50±6	45±9	30±1	198±20

* Adult male tissues were used, except where otherwise stated.
† Enzyme activities of erythrocytes are expressed as units/ml. whole blood. Plasma had no activity.
‡ Each value obtained from 7 pooled embryos.

Table 2. *Levels of enzymes of the direct oxidative pathway of carbohydrate metabolism in the mammary gland of the rat in pregnancy, lactation and involution*

	Enzyme activity (units/g. tissue at 20°*)			
		6-PG dehydrogenase		
State of mammary gland	G 6-P dehydrogenase	pH 9·0	pH 7·6	R 5-P breakdown
20 days pregnancy	86	92	50	197
5 days lactation	191	156	131	353
10 days lactation	1728	673	409	940
16 days lactation	2105	800	449	1080
21 days lactation	5452	1734	883	1300
2 days involution	50	93	45	147

* Enzyme activities of the abdominal mammary glands are corrected for retained milk by the method of Folley & Greenbaum (1947).

Table 3. *Levels of enzymes of the direct oxidative pathway of carbohydrate metabolism in tumours*

(Figures in parentheses represent number of animals.)

Tumour	Enzymic activity (units/g. tissue at 20°)			
	G 6-P dehydrogenase	6-PG dehydrogenase		R 5-P breakdown
		pH 9·0	pH 7·6	
Mouse sarcoma 37 (5)	58±11	60±5	54±2	—
Benzpyrene mouse sarcoma (3)	63±14	98±8	60±7	128±6
Benzpyrene mouse epithelioma (1)	126	137	84	289
Mouse squamous-celled carcinoma (3)	72±4	71±3	37±4	121±4
Spontaneous mammary adenocarcinoma C3H mice (1)	103	109	55	480
Mouse-lung carcinoma (1)	19	69	42	202
Spontaneous lymphoma C57 mice (1)				
Lymph nodes	290	140	97	378
Liver	222	153	129	344
Spleen	266	172	110	378
Walker rat carcinoma (1)	65	140	87	254
Spontaneous rat carcinoma (1)	127	193	148	306
A fowl tumour (3)	33±9	63±5	50±5	281±16

Breakdown of ribose 5-phosphate

Values for R 5-P breakdown, as measured by disappearance of pentose, are also included in Tables 1, 2 and 3. With a few exceptions, all the values are very much higher than the corresponding G 6-P and 6-PG dehydrogenase activities, indicating that the enzymes involved in pentose phosphate metabolism do not control rate-limiting steps in this direct oxidative pathway. At least two enzymes were involved in the disappearance of pentose: first, a pentose phosphate isomerase concerned in the interconversion of R 5-P and ribulose 5-phosphate (Horecker & Smyrniotis, 1950; Horecker, Smyrniotis & Seegmiller, 1951); and secondly, a pentose phosphate splitting enzyme, distinct from aldolase, which degrades ribulose 5-phosphate (Horecker & Smyrniotis, 1952; Horecker, Smyrniotis & Klenow, 1953; Racker, De La Haba & Leder, 1953). Pentose phosphate isomerase is clearly not rate-limiting in the present experiments, since ribulose was never detected (by absorption at 540 mμ. in the orcinol reaction) in any of the incubation mixtures at 30°, although it could sometimes be detected at 20°.

Resynthesis of hexose monophosphate from R 5-P (see Horecker, 1953) was found to occur in all the tissues and tumours examined, including skeletal and cardiac muscle. This is in contrast to Sable (1952) who found no hexosemonophosphate formation from R 5-P by aqueous muscle extracts, but only an accumulation of ketopentose 5-phosphate, and suggested that the enzymes catalysing this synthesis were absent from muscle.

DISCUSSION

The most interesting findings arising from this investigation appear to be the very high levels of activity of both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the adrenal cortex and in lactating mammary glands. The very low levels of activity in muscle, on the other hand, indicate that this pathway is quantitatively of relatively little importance in this tissue. The metabolic significance of these findings is, however, obscure, and warrants further experimentation, particularly in the cases of the adrenal cortex and of the mammary gland.

The very rapid increase in the levels of activity of G 6-P and 6-PG dehydrogenases in the mammary gland from the end of pregnancy to the end of lactation, followed by a fall in the level of activity in the involuting gland, is not peculiar to these particular dehydrogenases. The same pattern of activity has been demonstrated by Folley & Greenbaum (1947) in the case of arginase activity and milk production, and by Folley & French (1949) for the Q_{O_2} (μl. O_2/mg. dry wt./hr.) of mammary-gland slices. The pattern here described is different from that of alkaline phosphatase (Folley & Greenbaum, 1947), which increases during pregnancy and then remains constant throughout lactation. Since the weight of the mammary gland, after correcting for the milk content, is virtually constant in the rat throughout lactation (Folley & Greenbaum, 1947), these high values of G 6-P and 6-PG dehydrogenase activities appear to be connected with milk secretion rather than with growth of the gland. These results

also suggest that there might be active competition for glucose between the enzymes of this direct oxidative pathway and the pathway concerned with the synthesis of lactose, or with some other secretory activity of the gland.

Since one of the main functions of this direct oxidative pathway is probably to supply ribose 5-phosphate for incorporation into ribonucleic acid and coenzymes, it would be expected to play an important part in the metabolism of tumours and other rapidly dividing cells. The results of the present investigation, however, indicate that the G 6-P and 6-PG dehydrogenase activities of a variety of experimentally induced and spontaneous tumours all fall within the limits of the same activities in normal tissues, although no adequate controls are available. The present method of approach is limited, however, in that only the maximum enzymic capacities are determined, whereas in the intact cell other regulatory factors probably limit or control the activity of this pathway.

SUMMARY

1. Measurements of the activities of enzymes of the direct oxidative pathway of carbohydrate metabolism have been made in a variety of normal mammalian tissues and tumours.

2. Both glucose 6-phosphate (G 6-P) and 6-phosphogluconate (6-PG) dehydrogenase activities are very high in adrenal cortex, lactating mammary gland, lymphatic tissues in general (spleen, thymus, and lymph nodes), and in whole rat embryos in the early stages of development, and are strikingly low in muscle.

3. In mammary glands, the levels of activity of these two enzymes increase rapidly from the end of pregnancy to the end of lactation and then fall to very low levels in the involuting gland. The increases during lactation are approximately 60-fold for G 6-P dehydrogenase and 20-fold for 6-PG dehydrogenase.

4. The G 6-P and 6-PG dehydrogenase activities of a variety of experimentally induced and spontaneous tumours fall within the range of normal tissues and are relatively high in lymphomas and low in sarcomas.

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